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Differential activation of NF- κ B and nitric oxide in lymphocytes regulates *in vitro* and *in vivo* radiosensitivity

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Abstract

Lymphocytes are more sensitive to radiation *in vivo* than *in vitro*. However, the mechanism of this differential response is poorly understood. In the present study, it was found that the lipid peroxidation and cell death were significantly higher in lymphocytes following whole body irradiation (WBI) as compared to lymphocytes exposed to radiation *in vitro*. EL-4 cells transplanted in mice were also more sensitive to radiation than EL-4 cells irradiated *in vitro*. DNA repair, as assessed by comet assay, was significantly faster in lymphocytes exposed to 4 Gy radiation *in vitro* as compared to that in lymphocytes obtained from whole body irradiated mice exposed to the same dose of radiation. This was associated with increased NF- κ B activation in response to genotoxic stress and lesser activation of caspase in lymphocytes *in vitro* compared to *in vivo*. To explain the differential radiosensitivity, we postulated a role of nitric oxide, an extrinsic diffusible mediator of radiosensitivity that has also been implicated in DNA repair inhibition. Nitric oxide levels were significantly elevated in the plasma of whole body irradiated mice but not in the supernatant of cells irradiated *in vitro*. Addition of sodium nitroprusside (SNP), a nitric oxide donor to cells irradiated *in vitro* inhibited the repair of DNA damage and enhanced apoptosis (increased Bax to Bcl-2 ratio). Administration of L-NAME, a nitric oxide synthase inhibitor, to mice significantly protected lymphocytes against WBI-induced DNA damage and inhibited *in vivo* radiation-induced production of nitric oxide. These results confirm that the observed differential radiosensitivity of lymphocytes was due to slow repair of DNA due to nitric oxide production *in vivo*.

Keywords

Nitric oxide; Radiosensitivity; NF- κ B; Apoptosis; Proliferation; Lipid peroxidation

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Conflict of interest statement

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1. Introduction

Cellular damage by radiation can occur by direct transfer of energy to biomolecules as well as by free radical mediated indirect mechanisms. The generation of highly reactive oxygen species through radiolysis of water brings about oxidation of crucial cellular components leading to DNA strand breaks, peroxidation of lipid moieties in cellular membranes and protein oxidation [1,2]. Our previous studies clearly demonstrated that higher the levels of ROS generated, greater is the extent of radiation induced cell death and vice versa [3]. DNA is considered to be a crucial target for radiation-induced damage. Damage to DNA can lead to activation of various genes resulting in cell cycle arrest and recruitment of DNA repair enzymes to the damaged site [4,5]. The residual damage or misrepair can cause genomic instability and higher frequency of mutations resulting in either clonogenic cell death subsequent to several mitotic cycles or results into a transformed cell [6,7]. The other mechanism of radiation-induced cell death is by alterations occurring in the plasma membrane and further activation of different signal transduction pathways [8–10].

Higher radiosensitivity of tumor cells compared to normal cells is a very important requirement for success of cancer radiotherapy [11,12]. Multiple factors like quiescence, cell cycle stage, stage of differentiation, DNA repair and types of cells are known to influence radiosensitivities [13]. The concentration of oxygen also contributes to the radiosensitivity of cells [14].

It is widely accepted that cells irradiated *in vitro* are less radiosensitive than the cells irradiated *in vivo*. Both nitric oxide (NO) and activation of NF- κ B have been implicated in survival of irradiated cells [15–17]. The present study was focused on examining their contribution to the differences in the damage to lymphocytes and tumor cells under *in vitro* and *in vivo* irradiation conditions.

2. Materials and methods

2.1. Chemicals

Fetal calf serum (FCS) was obtained from GIBCO BRL. N^G -nitro-L-arginine-methyl-ester (L-NAME) was purchased from Calbiochem, USA. Carboxy fluorescein diacetate succinimidyl ester (CFSE) was procured from Molecular Probes, The Netherlands. RPMI 1640 tissue culture medium, propidium iodide (PI), tris base, EDTA, DMSO, agarose and sodium nitroprusside (SNP) were obtained from Sigma Chemical Company, USA. Dexamethasone was procured from Wockhardt Limited, Mumbai, India. PE labeled Bcl2 and Bax were purchased from Santacruz Biotechnology. Antibodies against caspase-3 and actin- β were procured from Cell Signaling Technology.

2.2. Animals

Eight to ten-week-old inbred C57BL/6 mice, weighing approximately 20–25 g and reared in the animal house of Bhabha Atomic Research Centre were used. The guidelines issued by the Institutional Animal Ethics Committee of Bhabha Atomic Research Centre, Government of India, regarding the maintenance and dissections of small animals were strictly followed. Ether anesthesia as permitted by BARC animal ethics committee was used in all our experiments. Animals were sacrificed by cervical dislocation.

2.3. Irradiation schedule

Whole body irradiation (WBI) and *in vitro* irradiation were carried out using Gamma Cell 220 irradiator machine (AECL, Canada). The dose rate as estimated by chemical dosimetry was 6.20 Gy/min. Animals were placed in perspex boxes and exposed to 4 Gy whole body

γ -irradiation (WBI). In all the experiments, mice were sacrificed 1, 4, 24 or 96 h after WBI. For *in vitro* experiments tumor (EL-4) cells and lymphocytes were resuspended in RPMI medium without FCS at a cell density of 5×10^6 cells/ml and exposed to 4 Gy radiation.

2.4. Preparation of cells

Spleen cells were obtained by squeezing the spleen through a nylon mesh in a petri plate containing RPMI medium. The RBC was lysed by brief hypotonic shock. Viable lymphocytes were enumerated by trypan blue dye exclusion. Peripheral blood mononuclear cells (PBMNCs) were obtained from the blood taken from lateral tail vein of live mice using a syringe (26.5 G). They were used for comet assay.

2.5. Analysis of lipid peroxidation following irradiation

Damage to lymphocyte cell membranes was assessed in terms of lipid peroxidation. The *in vitro* irradiated cells were plated in 24 well culture plates (2.5×10^6 cells/ml) in three replicates and were incubated at 37 °C in 5% CO₂ for 2, 4 or 24 h. Similarly, lymphocytes were obtained 2, 4 or 24 h after 4 Gy WBI. Cells isolated from unirradiated control mice served as control. Subsequently, lymphocytes were suspended in 300 μ l 10 mM potassium phosphate buffer pH 7.4, and 900 μ l TBA reagent (0.375% thiobarbituric acid (TBA), 0.25 M HCl, 15% trichloroacetic acid (TCA) and 6 mM EDTA) was added. The reaction mixture was incubated at 85 °C for 20 min, cooled to ambient temperature and centrifuged at $12,000 \times g$ for 10 min at 25 °C. Thiobarbituric acid reactive substances (TBARS) in the supernatant were estimated by measuring the absorption at 535 nm using Varian DMS 200 UV-Visible spectrophotometer. Lipid peroxidation values are expressed as nanomoles of TBARS per 2.5×10^6 cells.

2.6. Measurement of cell death

For estimation of cell survival, CFSE staining (1 μ M, 5 min, 37 °C) was carried out to study the cellular esterase activity following irradiation. CFSE is a non-fluorescent dye that is taken up by live as well as dead cells. The live cells contain esterase enzymes, which converts non-fluorescent CFSE to a fluorescent derivative carboxyfluorescein, which binds to amino groups in cellular proteins. The dead cells do not have esterase activity and hence remain non-fluorescent. After CFSE staining, 50,000 cells were acquired in a fluorescence activated cell sorter (FACS Vantage, Becton Dickinson Immunocytometry Systems, USA) and the fraction of non-fluorescent cells was gated using Cellquest[®] software and CFSE negative cells considered as dead cells.

For estimation of radiation induced death, lymphocytes were either exposed to 4 Gy radiation *in vitro* or obtained from mice after 4 Gy WBI and further cultured for 20 h *in vitro*. The unirradiated cells served as control. These cells were washed with 10 mM phosphate-buffered saline (PBS) and incubated overnight with 1 ml of staining solution containing 0.5 μ g/ml propidium iodide, 0.1% sodium citrate and 0.1% Triton X-100 [18]. A total of 20,000 cells were acquired on a Partec Cyflow flowcytometer and were analyzed using Flomax software. Undivided cells were in G1 phase of cell cycle (2n DNA content). The cells containing less than 2n DNA (sub-G1 population) represented the apoptotic cells.

2.7. Western blot analysis

Cell lysates were prepared from lymphocytes exposed to radiation *in vitro* or from lymphocytes obtained from WBI mice [19]. Briefly, the cells were washed with ice-cold phosphate-buffered saline and suspended in 0.1 ml lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 0.5 mg/ml benzamidine). The cells were allowed to swell

on ice for 15 min, after which 25 μ l of 10% nonidet P-40 was added and tubes were vortexed. The supernatants containing proteins from cytosolic fraction were collected by centrifuging the cells at 8000 rpm for 6 min at 4 °C. Proteins were fractionated by SDS-PAGE, electrotransferred on to a nitrocellulose membranes, probed with the appropriate antibodies, and detected by enhanced chemiluminescence (Roche, Germany). Ponceau staining of the blot or actin- β served as the loading control.

2.8. Immunofluorescence microscopy

Lymphocytes were seeded on glass cover slips at a density of 1×10^6 cells/ml after exposing them to radiation *in vitro* and *in vivo*. Cells were then fixed in 4% paraformaldehyde at 4 °C and then immunofluorescently labeled as described earlier [20] to visualize phosphorylated form of P65. DNA was stained with Hoechst33258. The coverslips were mounted in 80% glycerol in PBS containing 1,4-diazabicyclo[2.2.2]octane (DABCO) and observed with a 60 $\times$ water immersion objective in LSM510 scanning module (Carl Zeiss Microscopy, Jena GmbH, Germany) with a Krypton–Argon laser, coupled to an Orthoplan Zeiss confocal photomicroscope using a 488 nm laser line and a 510 band pass filter. The images were processed and analyzed using Adobe Photoshop software.

2.9. Evaluation of the effect of radiation on proliferation EL-4 tumor cell

The cell proliferation was measured by dye dilution using a flowcytometer. The EL-4 cells were stained with CFSE (20 μ M, 5 min, 37 °C) and washed three times with RPMI medium. For estimation of the effect of radiation on proliferation of tumor cells *in vitro*, CFSE labeled EL-4 cells were resuspended in RPMI medium (5×10^6 cells/ml) and exposed to 4 Gy γ -radiation. Cells were further cultured for 96 h at 37 °C in RPMI medium containing 10% FCS (complete medium, CM) in 5% CO₂. Untreated cells served as a control. The cell proliferation was measured by dye dilution using a flowcytometer. For estimation of the effect of irradiation *in vivo*, 1×10^7 CFSE labeled EL-4 cells were injected i.p. into C57BL/6 mice and the mice were exposed to 4 Gy WBI. The unirradiated mice bearing EL-4 tumor served as control. These mice were sacrificed after 96 h and the tumor cells were recovered by the peritoneal flush using 5 ml ice-cold RPMI medium. The percentage of daughter cells was determined using Cellquest[®] software.

2.10. Estimation of radiation induced DNA damage by single cell gel electrophoresis (SCGE) (Alkaline comet assay)

For estimation of DNA damage *in vivo*, peripheral blood mononuclear cells were isolated from whole body irradiated mice at different time intervals (5 min to 4 h) after irradiation and also from unirradiated control mice. For *in vitro* experiments, lymphocytes were resuspended in RPMI 1640 medium at a density of 5×10^6 cells/ml and exposed to 4 Gy radiation. The cells were further cultured for 5 min to 4 h at 37 °C. Unirradiated cells served as control. In one of the groups, SNP was added to the *in vitro* irradiated lymphocytes 30 min after irradiation. For studying the DNA damage in these cells alkaline single cell gel electrophoresis (comet assay) was carried out according to the method of Tice et al. [21]. The images were captured with high-performance GANZ (Model: ZC-Y11PH 4) color video camera. The quantification of the DNA strand breaks (comet tail length) in the stored images was done using the CASP software [22].

2.11. Estimation of radiation induced generation of nitric oxide

NO levels were estimated according to the method of Green et al. [23]. Blood was drawn from tail vein of live animals after respective treatments. Briefly plasma samples were obtained from control or whole body irradiated mice 2, 4 or 24 h after irradiation. For *in vitro* experiments, lymphocytes were irradiated *in vitro* (4 Gy). These cells were further

cultured in CM (5×10^6 cells/ml) for 2, 4 or 24 h at 37 °C in 5% CO₂ and the culture supernatants were collected. Plasma samples (100 µl) or undiluted culture supernatants (100 µl) were incubated with 100 µl of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-Naphthyl) ethylenediamine dihydrochloride (Sigma), 2.5% phosphoric acid) in distilled water. The absorbance was measured at 550 nm using an ELISA plate reader (Bio-Tek Instruments, USA). Amount of nitrite in each sample was calculated from a standard curve generated with known dilutions of sodium nitrite.

2.12. Intracellular antibody staining by flow cytometry

Lymphocytes (3×10^6 cells) were treated with SNP (200 µM) following exposure to 4 Gy radiation and were incubated for 24 h at 37 °C. Cultured cells were fixed with 4% paraformaldehyde for 10 min at room temperature and excess of paraformaldehyde was removed by washing with wash buffer (PBS containing 1% BSA). Cells were permeabilized by washing with PBST (PBS containing 0.02% Tween-20) thrice for 5 min each at room temperature followed by two washes with wash buffer and then incubated with antibodies against Bcl-2 or Bclxl for 30 min. Cells were then washed twice and analyzed on a Partec Cyflow flowcytometer using Flomax software.

2.13. Treatment with L-NAME, dexamethasone and SNP

For *in vitro* experiments, sodium nitroprusside (200 µM) was added in culture medium 30 min after irradiation of cells. For *in vivo* experiments, L-NAME (150 µg/mouse) or dexamethasone (200 µg/mouse) was injected i.p. 2 h before irradiation. Normal saline (0.9% sodium chloride) was injected as a vehicle control.

2.14. Statistical analysis

The statistical significance of the differences in respect of all parameters studied between *in vitro* irradiated cells and cells recovered from whole body irradiated mice was assessed by Student's 't'-test.

3. Results

3.1. Effect of radiation on lipid peroxidation, and viability of lymphocytes

Fig. 1a shows lipid peroxidation in lymphocytes as estimated by TBARS method. The *in vitro* irradiated (4 Gy) lymphocytes cultured for 2, 4 or 24 h did not show any enhancement in lipid peroxidation as compared to their unirradiated controls (Fig. 1a). However, the lymphocytes obtained from mice 2, 4 or 24 h after exposure to 4 Gy WBI treatment showed significantly elevated TBARS levels as compared to those from control untreated animals.

Fig. 1b shows the loss of esterase activity in lymphocytes 4 h after irradiation. The lymphocytes obtained from WBI exposed mice 0, 1 or 4 h after irradiation showed a significantly higher loss of esterase activity as compared to *in vitro* irradiated lymphocytes (Fig. 1b).

Radiation induced cell death as estimated by propidium iodide staining followed by flow cytometric analysis showed that there is a significant increase in sub-G1 population in lymphocytes obtained from WBI mice (4 Gy) as compared to lymphocytes irradiated *in vitro* and cultured further for 24 h (Fig. 1c and d).

Fig. 1e shows degradation of pro-caspase-3 under *in vitro* and *in vivo* irradiation conditions in lymphocytes after 24 h of culture. Lymphocytes isolated from WBI (4 Gy) treated mice showed higher activation of caspase-3 as evinced from degradation of pro-caspase-3 as

compared to that in lymphocytes irradiated (4 Gy) *in vitro* suggesting that radiation induced apoptosis is higher *in vivo* than *in vitro* (Fig. 1e).

3.2. Effect of *in vitro* and *in vivo* radiation on EL-4 tumor cell proliferation

Fig. 2a shows the comparison of flowcytometric histograms of EL-4 cells 96 h after *in vitro* irradiation and EL-4 cells taken from whole body irradiated mice 96 h after EL-4 transplantation. Fig. 2b shows percent daughter cells (M3 + M4) in both *in vitro* irradiated EL-4 cells and those isolated from WBI exposed tumor bearing mice. Even though the inhibition of proliferation was significant at 4 Gy *in vitro* irradiated EL-4 cells (65.8 + 0.69% daughter cells in 4 Gy irradiated group as compared to 72.5 + 3.56% daughter cells in control group) it was much more profound in the EL-4 cells that were stained with CFSE and injected into C57BL/6 mice and exposed to 4 Gy WBI (57.4 + 0.38% daughter cells in whole body irradiated group as compared to 81.2 + 0.55% daughter cells in control group) (Fig. 2b).

3.3. Effect of radiation on NO production *in vitro* and *in vivo*

NO production by lymphocytes exposed to 4 Gy γ -radiation *in vitro* was estimated in the culture supernatants 2, 4 or 24 h after irradiation. *In vivo* NO production was measured in the plasma obtained from mice 2, 4 or 24 h after 4 Gy WBI. Fig. 3 shows nitrite levels in the culture supernatants vis-à-vis in plasma obtained after *in vitro* or *in vivo* irradiation respectively. There was a marginal difference in nitrite levels in the culture supernatants obtained from lymphocytes irradiated *in vitro* at all the time points as compared to supernatants obtained from unirradiated lymphocytes. However, a significant increase in the nitrite levels was seen in the plasma obtained from mice 2, 4 or 24 h after WBI treatment as compared to that in the plasma from unirradiated mice (Fig. 3).

3.4. Effect of radiation on DNA damage *in vitro* and *in vivo*

Fig. 4a shows representative comet images of lymphocytes irradiated *in vitro* or *in vivo*. Fig. 4a and b depicts DNA damage and kinetics of repair in lymphocytes after *in vitro* irradiation or WBI as estimated by alkaline SCGE method. The tail length of the comet is a measure of DNA strand breaks [22]. Cells exhibited maximum DNA strand breaks 5 min after *in vitro* irradiation as shown by increase in tail length (Fig. 4b). Cells examined at later time points showed a decrease in the DNA damage, which is due to repair of DNA strand breaks. Most of the DNA damage (~90%) was repaired within first 60 min after *in vitro* irradiation. Almost complete repair was observed 4 h after irradiation. However, the DNA damage in lymphocytes isolated from mice at different time intervals following 4 Gy WBI showed slower repair (Fig. 4c) as compared to *in vitro* irradiated lymphocytes. About 40% of the DNA damage was repaired within first 60 min as compared to 90% repair in the *in vitro* irradiated cells (Fig. 4b and c). The lymphocytes isolated from mice 4 h after WBI showed ~80% DNA repair, which was still lower than the repair *in vitro*.

3.5. Effect of radiation on activation of NF- κ B *in vitro* and *in vivo*

Fig. 5a shows degradation of I κ B- α within 1 h after exposure of lymphocytes to radiation *in vitro*. However, the levels of I κ B- α were restored 4 h after of irradiation suggesting that maximum activation of NF- κ B was observed at early time points. Interestingly, I κ B- α degradation was not seen in lymphocytes taken from WBI mice at both 1 and 4 h. An immunocytochemical assay demonstrated the nuclear translocation of phosphorylated form of P65 1 h after lymphocytes exposed to *in vitro* radiation (2 Gy). Interestingly, there was no activation of P65 in lymphocytes at 4 h after 2 Gy *in vitro* irradiation. On the other hand P65 translocation was not detected in lymphocytes taken from WBI mice at both 1 and 4 h (Fig.

5b). These results corroborated with pattern of I κ B- α degradation as shown in Fig. 5a. Lymphocytes treated with TNF- α were used as positive control.

3.6. Effect of SNP treatment on radiation induced DNA damage

Fig. 6a shows the effect of addition of SNP to cells after *in vitro* irradiation. SNP was added to lymphocytes 30 min after irradiation. The extent of DNA damage as estimated by comet assay in irradiated cells was significantly higher as compared to that in unirradiated cells. Addition of SNP to unirradiated lymphocytes did not induce significant DNA damage. The radiation induced DNA damage was also not influenced by SNP addition 1 h after irradiation. However, after 4 h a significantly higher DNA damage was seen in irradiated cells treated with SNP as compared to only irradiated cells. This difference indicated that the irradiated cells repaired their DNA damage, but the irradiated cells treated with SNP failed to repair the DNA damage. This was further supported by the changes in the ratio of intracellular levels of Bax to that of Bcl-2 (Fig. 6b). The ratio of Bax to Bcl-2 determines the fate of the cell following exposure to ionizing radiation. This ratio increased when lymphocytes were exposed to *in vitro* radiation and cultured for 24 h suggesting an increase in apoptotic population. Addition of SNP to irradiated lymphocytes significantly enhanced the ratio of Bax to Bcl-2 as compared to irradiated lymphocytes cultured in the absence of SNP (Fig. 6b).

3.7. Effect of dexamethasone and L-NAME on radiation induced DNA damage and NO

Further support to the role of NO in enhancing the radiosensitivity of lymphocytes was provided using inhibitors of NO synthase. Fig. 7a shows the DNA damage in lymphocytes isolated from control and 4 Gy WBI exposed mice 1 and 4 h after irradiation. The mice were injected with dexamethasone (200 μ g/mice), L-NAME (150 μ g/mice) or saline 2 h prior to WBI treatment. Lymphocytes isolated from both dexamethasone as well as L-NAME treated mice did not show any increase in DNA damage as compared to that in lymphocytes isolated from saline injected mice. The lymphocytes isolated from saline treated mice 1 h after 4 Gy WBI treatment showed a significantly higher DNA damage as compared to lymphocytes isolated from unirradiated mice. The cells from dexamethasone treated WBI exposed mice showed slightly higher DNA damage as compared to only whole body irradiated mice. However, the lymphocytes from L-NAME treated WBI exposed mice showed a significantly less DNA damage as compared to only whole body irradiated mice. The lymphocytes isolated from dexamethasone or L-NAME or saline treated mice 4 h after WBI treatment showed a decrease in the total DNA damage due to repair. The cells from saline treated mice 4 h after WBI as well as cells from dexamethasone treated mice 4 h after WBI showed significantly higher DNA damage as compared to lymphocytes isolated from L-NAME treated mice 4 h after WBI.

Fig. 6b shows the plasma nitrite levels in control, 4 Gy WBI or L-NAME injected mice exposed to WBI. L-NAME was injected 2 h prior to irradiation and the plasma nitrite levels were estimated 24 h after exposure to WBI. The plasma nitrite levels in whole body irradiated mice were significantly higher than that in the control unirradiated mice. The L-NAME treated mice exposed to WBI showed significantly lower plasma nitrite levels as compared to only whole body irradiated mice (Fig. 7b).

4. Discussion

It has been generally accepted since long that radiation causes more deleterious effects to cellular systems *in vivo* as compared to *in vitro*. Availability of techniques to study the DNA damage and repair at single cell level allows us to revisit the differences from a mechanistic point of view. The present studies are an effort in that direction using lymphocytes which

are known to be sensitive to radiation. Extent of lipid peroxidation in lymphocytes from WBI exposed mice increased significantly over the control unirradiated mice but no such increase was observed when lymphocytes were exposed to *in vitro* radiation indicating higher oxidative damage *in vivo* as compared to *in vitro* (Fig. 1a). Earlier we had shown that higher oxidative damage is directly related to cell death in lymphocytes as well as tumor cells [3]. The differences in *in vitro* and *in vivo* radiation effects were also seen in terms of cell death, which was measured in terms of loss of esterase activity (viability), increase in sub-G1 population and increase in degradation of pro-caspase-3 (apoptosis) (Fig. 1b–e). Lymphocytes isolated from mice 1 h after exposure to WBI did not show significant loss of viability. But the lymphocytes isolated from mice 4 h after WBI treatment showed a significantly higher loss of viability (Fig. 1b). We have also observed (data not shown) that exposure of whole spleen *in vitro* to 4 Gy of radiation did not alter the esterase activity significantly as compared to that in cells irradiated *in vitro*. This suggested that the greater sensitivity of lymphocytes *in vivo* than *in vitro* may be attributable to extrinsic signals emanating from outside the spleen.

To examine whether this differential *in vitro* and *in vivo* radiation effect would also be seen in cells implanted into an animal's body, EL-4 tumor cells were injected into the peritoneal cavity of the mice. EL-4 cells are T lymphoma cells which can divide *in vitro* in culture and grow as ascitic tumors in MHC matched mice. The EL-4 cells recovered from whole body irradiated mice showed significantly higher inhibition of proliferation as compared to *in vitro* irradiated EL-4 cells (Fig. 2a and b). This further supports the conclusion that radiosensitivity of lymphocytes *in vivo* may be driven by a signal extrinsic to the lymphocytes, possibly a diffusible cytokine or chemokine or endocrine molecule.

One such candidate molecule is NO, a gaseous signaling molecule that serves as an endogenous regulator of blood flow and thrombosis, a principal neurotransmitter mediating erectile function, and a dominant mediator of inflammation and host defense. Multiple lines of evidence suggest that NO is a modulator of radiation response. First, generation of NO has been reported in leukocytes and in skin cells following WBI [15,16]. Second, soluble factors released from irradiated lymphocytes initiate a signaling cascade in unirradiated lymphocytes resulting in immunomodulation [24]. Third, radiosensitization of bystander tumor cells by NO has been reported [25]. Fourth, ionizing radiation and NO have been shown to synergistically promote apoptosis and growth inhibition of cancer [26]. Fifth, NO is a potent radiosensitizer of hypoxic tumor cells [25,27], a key determinant of radiation resistance. Lastly, NO donors such as nitroglycerin have been demonstrated to improve the effects of radiation therapy in solid cancers. Taken together, these reports support our hypothesis that NO may contribute to the greater radiosensitivity of lymphocytes *in vivo* as compared to *in vitro*. This hypothesis was further strengthened by the documentation of a significant increase in NO levels in the sera from whole body irradiated mice, but not in the supernatants of *in vitro* irradiated lymphocytes (Fig. 3).

NO can enhance radiation-induced damage by reacting with superoxide ions generated during radiolysis of water to produce peroxynitrite. Peroxynitrite in turn can induce cytotoxicity in cells by initiation of lipid peroxidation and direct inhibition of mitochondrial respiratory chain enzymes [28]. Alternatively, NO can inhibit the DNA repair machinery of the cells activated after irradiation [29] similar to its effect on DNA repair following UV and chemical induced damage [17]. In our studies, the kinetics of DNA repair was slower following radiation induced DNA damage *in vivo* as compared to that *in vitro* (Fig. 4b and c). This greater DNA repair efficiency *in vitro* was corroborated by the demonstration of greater NF- κ B activation *in vitro* than *in vivo*. NF- κ B is a key mediator of the cellular response to genotoxic stress downstream of DNA damage sensors. It counteracts apoptosis and allows cells to escape from lethal effects of DNA damage [30–32]. Interestingly, there

are reports that nitric oxide donating compounds are capable of suppressing DNA binding ability of NF- κ B [33]. In our study, nitric oxide which is generated in plasma may suppress NF- κ B under WBI conditions due to which I κ B- α degradation and P65 activation was not observed in lymphocytes obtained from whole body irradiated mice. On the other hand, there is no nitric oxide production under *in vitro* conditions, hence, NF- κ B activation was observed in lymphocytes. On the contrary, there are also reports which show that nitric oxide can activate NF- κ B [34].

NF- κ B, a prosurvival transcription factor, has been shown to be responsible for radioresistance of cells during radiotherapy by several investigators [35,36]. It was also shown that overexpression of an upstream kinase IKK led to radioprotection in mice [37]. A recent report by Burdelya et al. [38] showed that activation of NF- κ B by a peptide from flagellin offered protection against radiation induced lethality in mice and baboons. These results suggested that this transcription factor plays a critical role in survival of cells/organism. Our studies demonstrated activation of NF- κ B following *in vitro* irradiation (2 Gy) of lymphocytes but not in lymphocytes taken from WBI (2 Gy) treated mice (Fig. 5a and b). Activation of this transcription factor following *in vitro* irradiation may be responsible for the higher radioresistance under these conditions.

Consistent with the notion that inhibition of DNA repair contributes to radiosensitization of lymphocytes *in vivo*, L-NAME a competitive inhibitor of NO synthase protected lymphocytes against radiation induced DNA damage (Fig. 7a) and decreased plasma nitrite levels (Fig. 7b). Dexamethasone, a specific iNOS inhibitor did not protect these cells from radiation induced DNA damage (Fig. 7a). A direct involvement of NO in enhancing radiosensitivity of lymphocytes was confirmed when SNP, a NO donor, was added to *in vitro* irradiated cells and it impaired the repair of DNA damage in those cells (Fig. 6a). Addition of SNP to *in vitro* irradiated cells enhanced extent of apoptosis as evinced by an increase in the ratio of Bax to Bcl-2 as compared to cells irradiated *in vitro* in the absence of SNP (Fig. 6b). These results clearly demonstrated the role of nitric oxide in enhancing radiation induced cell death. Taken together with all the observations on nitric oxide, we infer that endothelial NOS may be contributing to the radiation induced elevation of plasma nitrite levels. However this assumption needs to be verified.

Our studies thus confirm that radiation induced damage is higher when cells receive radiation under *in vivo* conditions than *in vitro* and demonstrate that the more gradual repair of DNA and pronounced cell death were mediated by extrinsically derived NO. These results advance a role for exogenous NO-donating drugs (such as nitroglycerin) for enhancement of radiation induced depletion of lymphocytes in whole body irradiation protocols. Accidental whole body radiation exposure to 2 Gy leads to a radiation prodrome that can be managed conservatively, doses of 4 Gy or greater are associated with the development of a hematological syndrome that is often fatal without aggressive medical intervention requiring a bone marrow transplant. The dose of radiation, 4 Gy, which is used in our study, sits at the threshold of conservative management vs. aggressive management (possibly requiring bone marrow transplantation). To some extent, the biological insight gained from these experiments provide a conceptual framework for investigation of means to abrogate one potential mechanism of heightened radiosensitivity of lymphocytes and thereby, enhance survival following accidental exposure.

Our results suggest that NOS inhibitors may be therapeutic in the management of the hematopoietic syndrome following accidental whole body radiation exposure. It remains to be determined whether such agents can serve as post-exposure radioprotectors or mitigators of hematopoietic syndrome following accidental exposure.

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Fig. 1.

Radiation induced lipid peroxidation and cell death in lymphocytes and inhibition of proliferation in EL-4 cells. (a) Lymphocytes were exposed to 4 Gy γ -radiation *in vitro* and cultured for 2, 4, or 24 h. Lymphocytes were also isolated from mice 2, 4 or 24 h after 4 Gy WBI (*in vivo*). Lipid peroxidation in lymphocytes was estimated by TBARS method. Each value represents mean $\pm$ S.E.M. from three replicates and two such experiments were carried out. * $p < 0.05$ as compared to cells obtained from control mice. (b) Viability of lymphocytes as estimated by CFSE staining and flowcytometry. Lymphocytes were exposed to 4 Gy γ -radiation *in vitro*, or taken from mice 1 or 4 h after 4 Gy WBI and stained with CFSE. Fifty thousand cells were acquired in a flowcytometer and the percentage of non-fluorescent cells was calculated using Cellquest[®] software. Each value represents mean $\pm$ S.E.M. from four replicates and two such experiments were carried out. * $p < 0.01$, as compared to unirradiated cells and # $p < 0.01$ as compared to *in vitro* irradiated cells. (c) Flow cytometric histograms of propidium iodide labeled lymphocytes exposed to either *in vitro* radiation (4 Gy) or taken from WBI mice and cultured for 24 h. (d) Bar diagram showing percent cell death under *in vitro* and *in vivo* irradiation conditions as estimated by propidium iodide staining. Each value represents mean $\pm$ S.E.M. from three replicates and two such experiments were carried out. * $p < 0.05$, as compared to *in vitro* irradiated cells. (e) Apoptosis in lymphocytes

following *in vitro* irradiation and WBI. Lymphocytes were either exposed to 4 Gy radiation and cultured for 24 h *in vitro* or obtained from mice 4 h after 4 Gy WBI and further cultured for 20 h. Cells were lysed and proteins were fractionated on 10% SDS-PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using anti-caspase-3 antibody. Ponceau staining served as loading control.

Fig. 2. Comparison of *in vitro* irradiation and WBI induced suppression of EL-4 cell proliferation. (a) Flowcytometric histograms show dilution of CFSE fluorescence in EL-4 cells following *in vitro* irradiation as well as WBI treatment. EL-4 tumor cells were stained with CFSE and exposed to 4 Gy γ -radiation *in vitro*. Alternatively 10 million CFSE stained EL-4 cells were injected i.p. into C57BL/6 mice and the mice were exposed to 4 Gy WBI. Control and *in vitro* irradiated cells were cultured for 96 h. The EL-4 cells from WBI exposed mice were recovered by peritoneal flush 96 h after irradiation. Cell proliferation was monitored by CFSE dye dilution using flowcytometer. A total of 20,000 labeled cells were acquired. (b) The percentage of daughter cells (M3 + M4) in each histogram from (a). The data from a representative experiment is shown and two such experiments were carried out. The data

points represent mean $\pm$ S.E.M. of four replicates. * $p < 0.01$, as compared to unirradiated control cells, # $p < 0.01$ as compared to *in vitro* irradiated cells.

Fig. 3.

Radiation induced NO production *in vitro* and *in vivo*. NO was estimated in the culture supernatants of *in vitro* irradiated lymphocytes cultured for 2, 4 or 24 h or plasma obtained from mice 2, 4 or 24 h after WBI. Concentration of NO was estimated using Griess reagent. Each value represents mean $\pm$ S.E.M. from three replicates and two such experiments were carried out. * $p < 0.05$, as compared to control unirradiated mice.

Fig. 4. Radiation induced DNA damage and its repair *in vitro* and *in vivo*. (a) Representative images of comet assay performed on lymphocytes irradiated *in vitro* or obtained from whole body irradiated mice. (b) Lymphocytes were exposed to 4 Gy γ -radiation *in vitro* and aliquots of irradiated cells were drawn 5, 15, 30, 60 and 240 min after irradiation. DNA damage was estimated by alkaline single cell gel electrophoresis. Each value represents mean $\pm$ S.E.M. from 50 cells and two such experiments were carried out. $*p < 0.01$, as compared to unirradiated cells. (c) Lymphocytes were recovered from mice at different time intervals after 4 Gy WBI. DNA damage was monitored by single cell gel electrophoresis method. Each bar represents mean $\pm$ S.E.M. from 50 cells. The data from a representative experiment are shown and two such independent experiments with three replicates in each group were carried out. $*p < 0.01$, as compared to unirradiated mice.

Fig. 5. Differential activation of NF-κB during *in vitro* and *in vivo* irradiation conditions (a) Degradation of IκB-α following 1 and 4 h *in vitro* irradiation and WBI of lymphocytes. Cytosolic extracts were prepared from lymphocytes taken from WBI (2 Gy) treated mice and also from cells irradiated *in vitro*. Equal amount of proteins were fractionated on 10% SDS-PAGE, electrotransferred to nitrocellulose membrane and probed using anti-IκB-α and anti-β-actin. (b) Differential nuclear translocation of phospho-P65 in lymphocytes exposed to *in vitro* radiation (2 Gy) and in lymphocytes isolated from whole body irradiated mice. Then cells were stained with secondary antibody tagged with alexafluor and observed under confocal microscope. Lymphocytes were treated with TNF-α as a positive control for NF-κB activation. Green color represents phosphorylated form of P65 and blue color represents Hoechst stained nuclei. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 6.

Effect of SNP on radiation induced DNA damage in lymphocytes. (a) Lymphocytes were exposed to 4 Gy γ -radiation *in vitro* and SNP (200 μ M) was added 30 min after irradiation. Cellular DNA damage (comet tail length) was estimated by SCGE method 1 and 4 h after irradiation. Each value represents mean $\pm$ S.E.M. from four replicates and two such experiments were carried out. * $p < 0.01$, as compared to unirradiated respective controls. # $p < 0.01$, as compared to respective 4 Gy irradiated lymphocytes. (b) Lymphocytes were treated with SNP (200 μ M) following irradiation (4 Gy) and cultured for 24 h. Cells were labeled with PE tagged Bcl-2 or Bax antibodies and acquired on a flowcytometer. The ratio of Bax to Bcl-2 was estimated as an index of apoptosis. Two such independent experiments were carried out. * $p < 0.01$, as compared to 4 Gy irradiated lymphocytes.

Fig. 7.

Effect of L-NAME and dexamethasone on the repair of radiation induced DNA damage and radiation induced NO production. (a) Mice were administered dexamethasone (200 μg/mouse), L-NAME (150 μg/mouse) or saline (i.p.) 2 h prior to 4 Gy WBI treatment. DNA damage in lymphocytes from control and whole body irradiated mice was estimated by SCGE method 1 and 4 h after WBI. Each value represents mean tail length $\pm$ S.E.M. from 50 cells and two such experiments were carried out. (b) Mice were injected with L-NAME or saline and exposed to 4 Gy WBI. Plasma nitrite levels were estimated using Griess reagent 24 h after WBI treatment. Each value represents mean $\pm$ S.E.M. from four mice and two such experiments were carried. * $p < 0.01$, as compared to unirradiated group. # $p < 0.01$, as compared to whole body irradiated group.