Spatio-temporal changes in glutathione and thioredoxin redox couples during ionizing radiation-induced oxidative stress regulate tumor radio-resistance

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Abstract

Ionizing radiation (IR)-induced oxidative stress in tumor cells is effectively managed by constitutive and inducible antioxidant defense systems. This study was initiated to understand the relative contribution of different redox regulatory systems in determining the tumor radio-resistance. In this study, human T-cell lymphoma (Jurkat) cells were exposed to IR (4 Gy) and monitored for the spatio-temporal changes in cellular redox regulatory parameters. We monitored the changes in the levels of reactive oxygen species (ROS) (total, mitochondrial, primary, and secondary), thiols (total, surface, and intracellular), GSH/GSSG ratio, antioxidant enzyme activity viz. thioredoxin (Trx), Trx reductase (TrxR), glutathione peroxidase, and glutathione reductase with respect to time. We have also measured protein glutathionylation. We observed that tumor cells mount a biphasic response after IR exposure which can be divided into early (0–6 h) and late (16–48 h) responses in terms of changes in cellular redox parameters. During early response, constitutively active GSH and Trx systems respond to restore cellular redox balance to pre-exposure levels and help in activation of redox-sensitive transcription factor Nrf-2. During late response, increase in the levels of antioxidants GSH and Trx rescue cells against IR-mediated damage. We observed that disruption of either glutathione or thioredoxin metabolism led to partial impairment of ability of cells to survive against IR-induced damage. But simultaneous disruption of both the pathways significantly increased radio-sensitivity of Jurkat cells. This highlighted the importance of these two antioxidant pathways in regulating redox homeostasis under conditions of IR-induced oxidative stress.

Keywords: ionizing radiation, redox homeostasis, oxidative stress, radio-sensitivity, antioxidant

Introduction

Radiotherapy is one of the principal treatment modality for cancers. Exposure to IR induces DNA damage in tumor cells leading to cell death or loss of clonogenic survival [1]. Spectrum and type of damage induced by IR depend upon the quality and dose of IR. IR exposure induces around 850 pyrimidine lesions, 450 purine lesions, 1000 single-strand breaks (SSB), and 20–40 double-strand breaks (DSB)/cell/Gy in mammalian cells [2,3]. Low linear energy transfer (LET) radiation deposits 70% of energy by inducing isolated lesions that add up to oxidative burden of the cell. DNA damage induced per cell per Gy post low LET IR exposure is chemically identical to those formed by ROS [4]. Thus, type and severity of DNA damage induced in tumor tissue depend upon redox status of the cell. Considering the nature and type of IR-mediated damage responsible for tumor cell killing, role of ROS in influencing the outcome of radiotherapy cannot be overlooked. In fact, hyperactive antioxidant machinery is one of the strategies employed by tumor cells to overcome ROS-mediated DNA damage to evade IR-induced cell killing [5–7]. Therefore, strategies targeting antioxidant defense and redox homeostasis gain importance in light of the current scenario.

Cellular defense against oxidative stress and maintenance of redox homeostasis depends on the regulation of thiol–disulfide exchange [8,9]. Formation of reversible protein disulfides or protein-SSG mixed sulfides (glutathionylation) act as regulatory switches in response to alteration in cellular redox [10,11]. Cells harbor two major independent systems of redox buffers in the form of monothiol tripeptide “GSH” and dithiol protein “thioredoxin.” The first system contains glutathione (GSH) as reducing agent, which primarily functions in neutralizing reactive oxygen species (ROS) either directly or through glutathione peroxidase (GPx)-catalyzed reactions and protects protein-SH groups [12,13]. The second system comprises dithiol protein of low molecular mass, namely thioredoxin (Trx) which is a protein disulfide oxidoreductase and Trx reductase (TrxR) that reduces Trx [14]. Trx is one of the major determinants of cell fate as it regulates pro-apoptotic protein ASK1 [15,16]. Besides, it has an important role in regulating several redox reactions vital for cell survival under normal as well as stress conditions [17–19]. In case of low LET radiation, ability of tumor cells to effectively manage oxidative stress as compared with the surrounding normal tissue is one of the impediments in achieving good therapeutic gain during radiotherapy. This enforces a need for the development of novel modality for...
cancer therapies targeting oxidative metabolism with the aim to sensitize them to radiation. Holistic and compartmental responses of cellular antioxidant systems exert a systematic, spatio-temporally coordinated inducible response to oxidative insults such as IR. In our previous studies, we have demonstrated that under conditions of oxidative stress, inducible antioxidant responses controlled by Nrf-2 are active in murine T-cell lymphoma (EL4) cells. Previous studies from our laboratory have provided mechanistic insights into the possible correlation between tumor radio-sensitivity and redox state of lymphoma and prostate cancer cells [20,21]. Radio-resistant DU145 cells were shown to have relatively lower levels of ROS, higher levels of GSH, Nrf-2 and active Trx reductase as compared with radio-sensitive PC3 cells [21]. Fate of the cell exposed to IR is principally governed by DNA damage checkpoint proteins. A cell would undergo reproductive death/apoptosis or cell cycle arrest depending upon type of response orchestrated by DNA damage sensors viz. RAD50, NBS1, Mre11 or transducers viz. ATM, ATR, CHK1, CHK2, etc. [22–25]. Mitochondrial DNA is also considered as a sensitive target for cancer radiotherapy. Generation and persistence of ROS in the mitochondria after exposure of cells to IR make them vulnerable to DNA damage, mitochondrial dysfunction, and genomic instability [26–28]. A strong and effective antioxidant response against oxidative stress challenge can eliminate ROS which are potential threats to genome integrity [29]. Based on the present literature, it could be perceived that strategies targeting simultaneous disruption of glutathione and Trx metabolism in tumor cells may improve the outcome of radiotherapy treatment.

In order to appreciate the role of these factors in determining the intrinsic radio-resistance of tumor cells, experiments were carried out to study the radiation-induced spatio-temporal changes in different cellular redox parameters. It was found that a biphasic response was observed in the levels of ROS, thiols, activity of antioxidant enzymes, and other composite factors that together constitute antioxidant regulatory network. Our data indicate that it would be more pertinent and vital to target constitutive antioxidant defense armor of tumor cells in order to sensitize them to radiotherapy.

**Methods**

**Chemicals**

MitoSOX Red and Alexa Fluor 633 C5 maleimide (ALM) were purchased from Invitrogen (USA). Trx1 and TrxR1, ELISA sets and total thiol detection kit were purchased from Cayman Chemicals (Michigan, USA). T4 polynucleotide kinase and kinase buffers were purchased from New England Biolabs (Ipswich, MA). Oligonucleotide probe for Nrf-2 was purchased from Santacruz Biotechnology (Indianapolis, USA). shRNA plasmids for TrxR1, Trx1 and Trx2 were purchased from Origene (Rockville MD USA). All other chemicals used in our studies were obtained from reputed manufacturers and were of analytical grade.

**Cell culture and irradiation**

Jurkat (Human T-cell lymphoma) cells were obtained from Health Protection Agency Culture Collection, UK and cultured as per their recommendation in RPMI 1640 containing 10% FCS and 100 U/ml penicillin–streptomycin at 37°C in 5% CO₂/95% air incubator. Cultures were maintained in exponentially growing conditions with doubling time little less than 24 h and sub-cultured thrice in a week. Cells were suspended in medium and exposed to IR using a 6Co γ-irradiator at a dose rate of 1.319 Gy/min (Blood Irradiator 2000, BRIT, Mumbai). Dose rates and doses delivered were calculated on a regular basis by Radiation Safety and Systems Division (RSSD) of BARC using Fricke dosimetry.

**Isolation of human PBMC**

Human peripheral blood mononuclear cells (PBMC) were isolated from blood of healthy human donors by centrifugation with Ficoll-Paque PLUS (Amersham Pharmacia Biotech) and cultured in RPMI 1640 medium containing 10% fetal bovine serum or FBS and 100 U/ml penicillin–streptomycin at 37°C in CO₂ incubator. Cells were irradiated after isolation and examined for cell death after 24 h.

**Estimation of cell death**

The percent cell death was estimated using propidium iodide (PI) staining followed by flow cytometry (Cyflow, Partec) as described previously [30]. The pre G1 population represents cell death.

**ROS measurement**

Jurkat cells (2×10⁵/ml) were stained with 2’,7’-dichlorodihydrofluorescein diacetate (H₂DCF-DA) (20 μM) 485/535 nm for measurement of total cellular ROS (H₂O₂ and hydroxyl radicals) [31] or dihydroethidium (DHE) (10 μM) 480/610 nm for measurement of superoxide [32] or dihydrorhodamine 123 (DHR123) (10 μM) 488/535 nm or MitoSOX Red (5 μM) 510/580 nm for measurement of mitochondrial superoxide [33] as described previously [20] for measurement of primary ROS. For measurement of secondary ROS, cells were stained with different fluorescent probes after harvesting at indicated time points. Cells pre-treated with 5 μM diphenyleineiodonium (DPI) for 2 h were exposed to IR 4 Gy and subsequently harvested at respective time points, stained with H₂DCF-DA and acquired on a flow cytometer. Primary ROS levels were estimated by monitoring fluorescence measured using spectrofluorimeter (BioTek Multi-Mode Microplate Reader), whereas secondary ROS levels were estimated by measuring mean fluorescence intensity using flow cytometer.
Thiol estimation

Total thiols were estimated using thiol detection assay kit (Cayman chemicals) as per manufacturer’s instructions. Surface thiols were estimated using ALM, and intracellular thiol content was estimated using monochlorobimane (MCB) (Ex 375/Em 450) as described elsewhere [34]. MCB reacts rapidly and specifically with GSH through a reaction catalyzed by glutathione-S-transferase (GST) to produce a conjugate that fluoresces at 475 nm when excited around 406 nm. Recent studies have identified that labeling with MCB is not linearly proportional to GSH content but thiol content. Briefly, 4 x 10^3/ml cells were stained with 40 μM MCB and incubated at 37°C for 20 min. Cells were kept on ice to stop the GST-dependent staining reaction. Cells were then washed and maintained at 4°C. After MCB staining, cells were washed with ice-cold media and 5 μM ALM was added in cell suspension and incubated for 15 min on ice. Cells were then washed and kept at 4°C and were acquired on a flow cytometer. Unstained cells and individual stained cells were kept as control.

Estimation of GSH/GSSG ratio and Pr-SSG

GSH/GSSG ratio in Jurkat cells (1 x 10^6/ml) after IR exposure was measured by conventional enzyme cycling method [35]. Levels of protein-bound GSH were determined using method described elsewhere [36]. For protein (Pr)-SSG estimation, Jurkat cells (3 x 10^6/ml) were lysed and protein pellet was reduced by 1%NaBH4 and neutralized by 30% metaphosphoric acid. Supernatant and individual stained cells were kept as control.

Estimation of enzyme activities

Glutathione reductase and GPx activity was estimated as described elsewhere [37,38]. GR assay mixture consisted of 100 mM potassium phosphate buffer pH 7.2, 0.17 mM NADPH, 2.2 mM oxidized glutathione, and 0.5 mM desferrioxamine. GR assay mixture consisted of 100 mM potassium phosphate buffer pH 7.2, 0.5 mM EDTA and 0.17 mM NADPH, 1 mM insulin, and 100 nM purified calf thymus TrxR and cell extract in final volume of 1 ml. After incubating at 37°C for 20 min, the reaction was stopped by adding 500 μl of stopping solution containing 0.2 M Tris–HCl, 6 M guanidine–HCl, and 1 mM 5,5’-dithiobis (2-nitrobenzoic acid) (DTNB), and absorbance was measured at 412 nm. For Trx activity measurement, cell lysate was added in 0.2 M potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 0.4 mM NADPH. Reactions were started by adding 2 mM DTNB and were followed spectrophotometrically at 412 nm. Similarly, cell lysates were treated with 1 μM auranoﬁ n and incubated at 37°C for 30 min to inhibit TrxR. The change in absorbance was monitored at 412 nm. Difference between the two readings was used to calculate the enzyme activity.

Glucose-6-phosphate dehydrogenase activity was estimated as described elsewhere [41]. Briefly, glucose-6-phosphate dehydrogenase (G6PD) buffer was prepared containing bovine serum albumin or BSA, NADP+, and glucose-6-phosphate. Cell lysates were added to the buffer and change in absorbance was monitored at 340 nm.

Electrophoretic mobility shift assay

Jurkat cells (2 x 10^6/ml) exposed to IR 4 Gy were harvested at different time intervals and nuclear extracts were subjected to electrophoretic mobility shift assay (EMSA) [42,44] by incubating with 32P-end-labeled (5’-TGTTACCCCTTTCACGTGGAG-3’) Nrf-2 oligonucleotide as described earlier [20]. EMSA was performed by incubating 8 μg of nuclear protein with 16 fmol of 32P-end-labeled, Nrf-2 oligonucleotide in the presence of 2 μg of poly(2’-deoxyinosinic-2’-deoxyctydylidic acid) in binding buffer (20 mM HEPES, pH 7.9, 0.4 mM EDTA, 0.4 mM dithiothreitol or DTT, and 5% glycerol) for 30 min at 37°C. The DNA–protein complex formed was separated from free oligonucleotide on 7.6% native polyacrylamide gels using buffer containing 50 mM Tris, 6 M guanidine – HCl, and 1 mM 5,5′-dithiobis (2-nitrobenzoic acid) buffer was prepared as described elsewhere [41]. Thioredoxin (Trx) and TrxR activity was measured as described elsewhere [39,40]. Trx activity was determined by insulin micro-method of reduction. All assay tubes contained 0.26 M HEPES, pH 7.6, 10 mM EDTA, 2 mM NADPH, 1 mM insulin, and 100 mM purified calf thymus TrxR and cell extract in final volume of 100 μl. After incubating at 37°C for 20 min, the reaction was stopped by adding 500 μl of 3-phosphate dehydrogenase (G6PD) buffer was prepared containing bovine serum albumin or BSA, NADP+, and glucose-6-phosphate. Cell lysates were added to the buffer and change in absorbance was monitored at 340 nm.

ELISA and RT-PCR

Jurkat cells (1 x 10^6/ml) exposed to IR 4 Gy were harvested at different time intervals and whole cell/nuclear extracts were probed for levels of Trx1/TrxR1 by ELISA using antibody-coated wells provided by Cayman Chemicals as per manufacturer’s protocol. Jurkat cells (1 x 10^6/ml) were processed for RNA isolation, cDNA preparation, and RT-PCR for Trx1, Trx2, and β-actin as described previously [30]. Table I shows primer sequences of Trx1, Trx2, and β-actin.

Table I. Primer sequences of Trx1, Trx2, and β-actin.

| Gene     | Sequence               |
|----------|------------------------|
| Trx1     | Forward: 5’GTGAGACAGATCGAGAGCAAG 3’  |
|          | Reverse: 5’CTGTTGAGGATCTACACTA 3’  |
| Trx2     | Forward: 5’CTGTTGAGGATCTACACTA 3’  |
|          | Reverse: 5’TCTCCTAATATTCGAGATCTA 3’ |
| β-actin  | Forward: 5’CATGTCATTTGCAATTCGG 3’  |
|          | Reverse: 5’CTCCTAATATTCGAGATCTA 3’ |
**Transfection**

Jurkat cells (2 × 10⁶/ml) were incubated in R buffer (Invitrogen) containing 5 μg shRNA plasmid DNA for TrxR1/Trx1/Trx2/scrambled sequence at RT for 10 min. Cells were electroporated using Neon Electroporator (Invitrogen) at pulse voltage of 1325 volts, pulse width 10 ms, and pulse number 3 at a cell density of 2×10⁶ cells/ml. Cells were cultured for 48 h and observed under fluorescence microscope for transfection. Knockdown of TrxR1 was confirmed by ELISA, whereas knockdown of Trx1/Trx2 was confirmed by RT-PCR.

**Statistical analysis**

Data are presented as mean ± standard error of mean (SEM). Statistical analysis was done using Student’s t-test by Microcal Origin 8.0 and by one-way ANOVA using Microsoft Office Excel. *refers to p < 0.05 as compared with control, # refers to p < 0.05 as compared with irradiated cells.

**Results**

**Jurkat cells but not human PBMC displayed resistance to IR-induced apoptosis**

Jurkat cells exposed to IR 4 Gy were cultured for different time periods (16 h, 24 h, 36 h, and 48 h) and analyzed for cell death by flow cytometry. It was observed that IR exposure led to 30% increase in cell death over control at 48 h (Figure 1A and B), whereas at 16 h, cells were arrested in S + G2/M phase. To compare the effect of similar dose of IR on the corresponding normal cells, human PBMC were exposed to IR 4 Gy and cultured for 24 h. Exposure to IR led to −70% cell death over control in human PBMC at 24 h. On the contrary, only −5% cells underwent cell death at 24 h in Jurkat cells (Figure 1C and D). Since radiotherapy treatment regimens involve exposure to dose fractions of radiation, we monitored the effect of dose fractionation on cell death in Jurkat cells. Cells were exposed to either 6 Gy acute or fractionated (3 × 2 Gy) dose of IR (Figure 1E and F). Exposure to fractionated doses significantly reduced cell death as compared with acute dose.

**Exposure to IR induced primary and secondary oxidative burst**

We monitored instantaneous changes in intracellular ROS levels after radiation exposure. H₂DCFDA, DHE, and MitoSOX Red are convenient probes for measuring general aspects of intracellular oxidative stress and redox status. There was significant increase in primary ROS levels (H₂O₂, hydroxyl radicals, and cytosolic and mitochondrial superoxide) immediately after IR exposure (Figure 2A and B). We measured total (H₂O₂, hydroxyl, and superoxide radicals) as well as mitochondrial ROS levels (superoxide) at different time points post IR exposure (secondary radicals). At early time points (30 min to 6 h), we observed a transient increase in total cellular ROS levels (Figure 2C) but significant increase in ROS levels (secondary radicals that include radicals generated by activation of NADPH oxidase, xanthine oxidase, and also some of the long-living lipid, protein, and DNA radicals) was evident at late time points like 16 h and 24 h post IR exposure (Figure 2D). Apart from IR-induced oxidative stress, ROS are also formed in cells as a by-product of metabolic processes such as electron transport chain from mitochondria, NADPH oxidase, and xanthine oxidase [43]. Pharmacological inhibitor of NADPH oxidase significantly abolished IR-induced increase in ROS at 16 and 24 h (Figure 2E) suggesting that NADPH oxidase may be the source of secondary ROS generation. It is noteworthy that the elevation in total ROS levels was not accompanied with elevation in mitochondrial ROS at early time points. However, increase in mitochondrial ROS levels was observed only after 36 h onwards (Figure 2F and G). Interestingly, remarkable increase in total ROS was observed at 36 and 48 h post-exposure, whereas significant increase in mitochondrial ROS was observed only at 48 h.

**Cellular thiols were differentially modulated upon IR exposure**

Endogenous oxidative stress can lead to changes in thiol levels affecting cellular redox balance. Hence, we measured levels of total thiols, surface thiols, and intracellular thiols (GSH) in response to IR exposure. An increase in intracellular ROS levels was expected to deplete the cellular thiols. However, it was observed that total and intracellular thiols increased significantly at early as well as late time points after IR exposure. The increase in thiols may help them to cope with the oxidative burden. But levels of both these thiols decreased below control at 48 h (Figure 3A–D). Levels of surface thiols on Jurkat cells increased till 6 h and were significantly dropped below control from 16 h up to 48 h (Figure 3E and F).

**Thioredoxin system was involved in activation of pro-survival transcription factor Nrf-2 after IR exposure**

Keap-1, a negative regulator of pro-survival transcription factor Nrf-2, is susceptible to inactivation due to oxidative stress [44]. We observed a significant increase in DNA binding of Nrf-2 at 6 h post IR exposure (Figure 4A). Band of Nrf-2 was confirmed with the use of cold and mutant oligonucleotides (Figure 4B). Binding of transcription factors to DNA is a Trx-dependent process [45–47]. We observed that IR exposure significantly increased levels (Figure 4C) and activity of Trx1 (Figure 4D) in nucleus at 6 h which may be responsible for the DNA binding of Nrf-2. Activity of TrxR also increased significantly in nucleus at 6 h post IR exposure (Figure 4E). These results revealed that time point at which there was enhanced DNA binding of Nrf-2 coincided with increased activity of Trx/TrxR in the nuclear extracts. This
indicated the association of Trx system in assisting activation of oxidative stress-induced DNA binding of Nrf-2.

Glutathione antioxidant regulatory network restored the cellular redox balance after IR exposure

Thiols alone do not completely represent the cellular redox status as they are associated with disulfides inside cells. Therefore, we measured ratio of GSH to GSSG, an indicator of cellular redox status. Exposure to IR led to decrease in GSH/GSSG ratio at 30 min and 1 h post exposure but the ratio significantly increased at 3 h (Figure 5A) indicating that cells were under oxidative stress at early time points. Our results were in agreement with those reported by Jayakumar et al, wherein the magnitude and kinetics of recovery from oxidative stress in terms of restoration of GSH/GSSG ratio post IR exposure is indicative of tumor radio-resistance [21]. GSH/GSSG ratio increased at 16 and 24 h after radiation which was restored to basal levels at 36 h and was less than control at 48 h (Figure 5B). Conversion of GSH to GSSG and vice versa is regulated by GPx and glutathione reductase (GR), respectively. Hence, we measured activity of these enzymes and observed that GPx activity was higher at 30 min suggesting utilization of GSH for neutralization of ROS. However, GR activity was less than control at initial time points and was restored to
Figure 2. IR exposure induced a biphasic response in terms of ROS generation: (A, B) Jurkat cells \((1 \times 10^5)\) were stained with \(\text{H}_2\text{DCF-DA} \, (20 \mu\text{M})\) or \(\text{DHE} \, (10 \mu\text{M})\) or \(\text{DHR123} \, (10 \mu\text{M})\) or \(\text{MitoSOX Red} \, (5 \mu\text{M})\) at 37°C for 20 min and cells were exposed to IR 4 Gy. Fluorescence was measured using spectrofluorimeter. Fluorescence estimated in arbitrary units was normalized by comparing it to respective controls. Data represents normalized fluorescence ± SEM from four replicates, and three such independent experiments were carried out. (C) Jurkat cells \((2 \times 10^5)\) exposed to IR were harvested at indicated time points and stained with either \(\text{H}_2\text{DCF-DA} \, (C–E)\) or \(\text{MitoSOX Red} \, (F, G)\) and fluorescence was measured using flow cytometer. (E) Cells were pre-treated with diphenyleneiodonium and subsequently exposed to IR, harvested at indicated time points, stained with \(\text{H}_2\text{DCF-DA}\), and acquired on flow cytometer. Bar graph represents normalized mean fluorescence intensity with the respective controls. Data set represents normalized fluorescence intensity ± SEM from three replicates and two such independent experiments were carried out (C, E). One-way ANOVA was performed to calculate statistical significance between groups along with Student’s t-test for paired comparison (E). Data set represents normalized fluorescence intensity ± SEM from 5 replicates from two independent experiments (F). Data set represents normalized fluorescence intensity ± SEM from 6 replicates from two independent experiments (D, G). *p < 0.05 as compared with control cells and #p < 0.05 as compared with irradiated cells.
normal afterward (Figure 5C). Interestingly, GPx activity was lower than control at 16 and 24 h. At later time points, activity of GR increased whereas activity of GPx was comparable to control (Figure 5D). Under oxidative stress conditions GSH is known to be consumed for scavenging ROS or protecting protein-SH groups [48]. Our findings revealed that there was utilization of GSH concomitant with elevated ROS levels (as seen in Figure 2A) and increased protein glutathionylation (Figure 5E). Interestingly, protein-SSG levels also increased at 16 h (Figure 5F). Glucose-6-phosphate dehydrogenase is a rate-limiting enzyme in pentose phosphate pathway. It is involved in synthesis of reducing equivalent NADPH and pentose sugars required in DNA synthesis [49]. It was observed that the activity of this enzyme increased significantly at 16 and 24 h, but decreased below control at 48 h (Figure 5G).

**Thioredoxin system complemented GSH system in restoring cellular redox homeostasis**

Thioredoxin (Trx) system is also involved in regulating cellular redox balance in oxidative stress conditions. Activity, levels, and oxidation status of Trx play an important regulatory role in cell fate upon IR exposure [50,51].

Figure 3. Exposure to IR led to differential and site-specific modulation of thiols: (A, B) Jurkat cells (2 × 10⁶) exposed to IR were harvested at indicated time points and cell pellet was used to estimate total thiol levels. Bar graph represents total thiol concentration (mM/mg protein). Data represents mean ± SEM from four replicates, and two such experiments were carried out (A). Data set represents mean ± SEM from 8 replicates from two experiments (B). (C–F) Jurkat cells (2 × 10⁵) exposed to IR were harvested at indicated time points, stained with MCB and ALM, and acquired on flow cytometer. Bar graph represents normalized mean fluorescence intensity with the respective controls. Data represents mean ± SEM from four replicates, and three such experiments were carried out. *p < 0.05 as compared with control cells.
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Figure 4. Trx system helped in DNA binding of Nrf-2: (A, B) Jurkat cells ($2 \times 10^6$) exposed to IR 4 Gy was harvested at indicated time points. Nuclear extracts were prepared and probed for Nrf-2. Gel shift assay was performed with Nrf-2 wild-type and mutant consensus sequences. Representative image is shown. Three independent experiments were performed. (C) Nuclear extracts from Jurkat cells exposed to IR 4 Gy were probed to detect levels of Trx1 by ELISA. Bar graph represents concentration of Trx1 (pg/ml). Data represents mean ± SEM from six replicates from two independent experiments. (D, E) Trx and TrxR activity was determined in the nuclear extracts of Jurkat cells exposed to IR. Bar graph represents nmol/mg protein for (D) and mU/mg enzyme activity for (E). Data represents mean ± SEM from three replicates, and two such experiments were carried out (D). Data set represents mean ± SEM from 5 replicates from two independent experiments (E). *$p < 0.05$ as compared with control cells.

Hence, we measured total cellular activity of Trx in response to IR exposure. Our findings have demonstrated that activity of Trx significantly increased at 30 min, 1 h, 16 h, and 24 h post IR exposure (Figure 6A and B). TrxR activity in whole-cell lysate also increased significantly at 30 min and 24 h post IR exposure (Figure 6C and D).

Blocking the constitutive antioxidant defense systems enhanced radio-sensitivity of Jurkat cells

To investigate the effect of blocking GSH or Trx network on cellular redox status, we measured ROS levels after inhibiting GSH or Trx. Glutathione synthesis was inhibited using...
Figure 5. Glutathione system responded to manage IR-induced oxidative stress: (A, B) Jurkat cells (1 x 10^6) exposed to IR was harvested at indicated time points and cell pellet was processed to estimate cellular concentration of GSH and GSSG. Bar graph represents (%) GSH/GSSG. Data represents mean ± SEM from five replicates from two independent experiments (A) and 9 replicates from three independent experiments (B). (C, D) Jurkat cells (2 x 10^6) were harvested at indicated time points and cell pellet was processed to determine GR and GPx activity using spectrophotometer. Bar graph represents mUnit/mg enzyme activity. Data represents mean ± SEM from three replicates and two such experiments were carried out. (E, F) Jurkat cells harvested at indicated time points were processed for Pr-SSG estimation and concentration was determined using spectrophotometer. Bar graph represents GSH (mM) bound to protein. Data represents mean ± SEM from 6 replicates from two independent experiments. (G) Jurkat cells (2 x 10^6) were harvested as mentioned above and cell pellet was processed G6PD activity. Bar graph represents G6PD activity in mUnit/mg. Data represents mean ± SEM from 6 replicates from two independent experiments. *p < 0.05 as compared with control cells.

buthionine sulfoximine (BSO), an inhibitor of glutamate cysteine ligase catalytic subunit (GCLC), which is an enzyme catalyzing the rate-limiting step in GSH synthesis. We inhibited TrxR using pharmacological inhibitor, auranofin. Depletion of GSH or inhibition of TrxR led to significant increase in ROS levels in the absence of radiation which was further enhanced after radiation exposure (Figure 7A and B). Increased ROS levels may increase tumor radio-sensitivity.
and hence we investigated effect of GSH and/or Trx depletion on radio-sensitivity of Jurkat cells. We used shRNA plasmids of TrxR1/Trx1/Trx2 to knock down gene expression of the respective genes prior to exposure to IR. shRNA plasmid showing maximum reduction in protein levels of TrxR1 or mRNA levels of Trx1/Trx2 was selected for transfection experiments (Supplementary Figure 1 A–C to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1056180). Our findings have demonstrated that inhibiting either GSH or TrxR1 did not alter cell survival under basal conditions. When cells were exposed to IR, there was a significant increase in cell death as compared with IR alone (Figure 7C and D). When GSH synthesis was blocked along with knockdown of TrxR1, it displayed a synergistic effect in cell death. Interestingly, when Trx1/2 knockdown cells were exposed to IR they also displayed increased cell killing. Especially, when Trx2 knockdown cells were exposed to IR, cells displayed significant increase in cell death as compared with Trx1 (Figure 7E and F).

**Discussions and conclusion**

A single DNA double-strand break left unrepaired after exposure to IR is believed to cause either cell death or genome instability. Differential radio-sensitivity of normal and tumor cells depend upon the inherent capacity to prevent or repair DNA damage [52,53]. There are several studies showing that the extent of DNA damage is different between resistant and sensitive phenotypes upon exposure of cells to the same dose of radiation [21]. Since ROS are the principal mediators of IR-induced DNA damage, prevention of this damage and manifestation of radio-resistance depends upon efficiency of antioxidant defense mechanism inside the cell. Survival of cells exposed to IR depends upon how efficiently cellular redox imbalance is restored to homeostatic conditions. Restoration of redox homeostasis depends on efficiency of constitutive antioxidant defense systems and their interplay with inducible oxidative metabolism [54,55].

Our results revealed that, depending on time after IR exposure, Jurkat cells displayed cell cycle arrest in S/G2/M phase (16 h) or apoptosis in (48 h) (Figure 1A and B). Interestingly, it was observed that IR-induced cell death in human PBMC was significantly higher as compared with Jurkat cells, suggesting that these cells were highly radiosensitive (Figure 1C and D). Jurkat cells underwent lower apoptotic death after exposure to fractionated dose of 6 Gy (3 × 2 Gy) than acute exposure of 6 Gy (Figure 1E and F). These experiments revealed that Jurkat cells exhibited resistance to IR-induced apoptotic death and to investigate the role of antioxidant network, we have categorized the radiation response of Jurkat cells into early (0–6 h) and late (up to 48 h) responses.

Our present results revealed that Jurkat cells displayed a biphasic response in terms of ROS (H₂O₂, hydroxyl,
Table 1: DNA Content

| Condition               | Count     |
|-------------------------|-----------|
| shTrxR + BSO + 4 Gy     | AF + Rad 4 Gy   |
| Mock                    | 1000       |
| shScr + BSO + 4 Gy      | AF + Rad 4 Gy   |
| Mock + Rad 4 Gy         | 1000       |
| shTrxR + BSO            | AF + BSO     |
| Mock                    | 1000       |
| shScr + BSO             | AF + BSO     |
| Mock + Rad 4 Gy         | 1000       |
| shTrxR 1                | 1000       |
| shScr                  | 1000       |
| shTrx 1                | 1000       |
| shScr                  | 1000       |
| Mock                    | 1000       |
| shTrx 2                | 1000       |
| shScr                  | 1000       |
| shTrx 1                | 1000       |
| shScr                  | 1000       |
| Mock                    | 1000       |
| shTrx 2                | 1000       |
| shScr                  | 1000       |
| shTrx 1                | 1000       |
| shScr                  | 1000       |
| Mock                    | 1000       |
| shTrx 2                | 1000       |
| shScr                  | 1000       |
| shTrx 1                | 1000       |
| shScr                  | 1000       |
| Mock                    | 1000       |

Figure 7. Blocking constitutive antioxidant defense systems alone or in combination increased sensitivity to IR induced cell death: (A, B) Jurkat cells \((1 \times 10^6)\) were stained with MitoSOX Red and treated with auranofin \((1 \mu M)\) for 30 min or treated with BSO \((1 \text{mM})\) for 24 h and stained with H₂DCFDA. Cells were exposed to IR 4 Gy and fluorescence was measured using spectrofluorimeter. Bar graph represents normalized fluorescence with the respective controls. Data set represents normalized fluorescence ± SEM from 6 replicates from two independent experiments. (C, D) Jurkat cells \((1 \times 10^6)\) were electroporated using shRNA plasmids of TrxR1/scrambled sequence and cultured for 48 h or treated with BSO for 24 h. Cells were subsequently exposed to IR 4 Gy and cultured for 48 h. Cells were harvested, stained with PI, and acquired on flow cytometer for Pre-G1 peak analysis. Representative flow cytometric histograms are shown. Bar graph represents percent cell death. Data represents mean ± SEM from three replicates and three such experiments were carried out. (E, F) Jurkat cells \((1 \times 10^6)\) were electroporated using shRNA plasmids of Trx1/Trx2/scrambled sequence and cultured for 48 h. Cells were exposed to IR 4 Gy and analyzed for Pre-G1 population by PI staining followed by flow cytometry. Representative flow cytometric histograms are shown. Bar graph represents percent cell death. Data represents mean ± SEM from three replicates and three such experiments were carried out. One-way ANOVA was performed to calculate statistical significance between groups along with Student’s t-test for paired comparison. *\(p < 0.05\) as compared with control cells, #\(p < 0.05\) as compared with irradiated cells.

cytosolic, and mitochondrial superoxide radicals) generation. Apart from initial oxidative burst (Figure 2A and B) there was a second wave of increased ROS at late time points (secondary radicals). The source of secondary ROS could be reaction of primary reactive ROS with cellular macromolecules or by-product of cellular metabolism [56]. Interestingly, pre-treatment with NADPH oxidase inhibitor abolished this second wave of ROS, suggesting...
that IR exposure activated endogenous ROS generation (Figure 2E). Mitochondrial ROS (superoxide radical) was evident only at 36 and 48 h post IR exposure (Figure 2G). Ability of mitochondria to cope with initial phase of oxidative stress may also contribute to radio-resistance. When levels of total and mitochondrial ROS increased significantly, apoptosis was induced in Jurkat cells.

Other than ROS, cellular thiols also play an important role in determining cellular redox state. Total cellular thiols comprise small-molecular-weight thiols, reactive protein thiols, and surface thiols. They determine total antioxidant capacity of a cell by maintaining cellular thioldisulfide balance at distinct cellular locations [57–59]. Our findings revealed that there was a concomitant increase in intracellular ROS levels (Figure 2D) and concentration of total and intracellular thiols (Figure 3B and D) after IR exposure. This simultaneous increases in the load of oxidants (ROS) and antioxidants (thiols) compensate each other to maintain redox harmony [60]. But levels of surface thiols dropped significantly during late response which could be attributed to ROS generation at the vicinity of cell membrane (Figure 2B). This increase in ROS was abolished upon incubation with NADPH oxidase inhibitor indicating the role of this enzyme in second wave of ROS generation after exposure to radiation (Figure 2C).

IR exposure led to nuclear translocation of redox-sensitive transcription factor Nrf-2 (Figure 4A and B). Nuclear accumulation and DNA binding of Nrf-2 requires phosphorylation at Ser-40 residue. DNA binding of Nrf-2 is a Trx-dependent process. We observed that levels and activity of components of Trx system increased significantly post IR exposure at the time point when there was enhanced DNA binding of Nrf-2 (Figure 4C–E). These results and literature reports suggest that Trx system might have provided assistance in DNA binding of Nrf-2 after IR exposure in Jurkat cells. Nrf-2 executes transcription of a number of genes involved in mediating antioxidant response against oxidative stress.

Glutathione and Trx systems are the two major constitutive antioxidant regulatory systems inside cells that compensate and complement with each other in maintenance of redox homeostasis [61,62]. GSH is the primary cellular redox buffer which is utilized during oxidative stress for neutralization of ROS and protection of protein thiols to keep them in reduced state [63]. Consistent with the findings of Jayakumar et al. that GSH/GSSG ratio in radio-resistant cells remained relatively unaltered post IR exposure, our results revealed that initial decrease in ratio of GSH to GSSG was restored back to control levels at late time points [21]. Our study observed that there was a decrease in GSH/GSSG ratio during early response, suggesting utilization of GSH to counter IR-induced oxidative stress (Figure 5A). During late response, there was an increase in GSH/GSSG ratio at 16 and 24 h (Figure 5B). This may be due to endogenous synthesis of GSH, mediated by Nrf-2 that regulates GSH synthesizing enzyme (GCLC). At 48 h, GSH/GSSG ratio again dropped below control levels as there was an increase in ROS (Figures 5B and 2D). Changes in GPx and GR activity also corroborated well with restoration of GSH/GSSG ratio during early and late responses (Figure 5C and D). Interestingly, at early and late time points there was an increase in protein glutathionylation that protects reactive protein thiols from oxidative modification (Figure 5E and F). G6PD is a metabolic enzyme regulated by Nrf-2 [64] that catalyzes rate-limiting step in NADPH synthesis which is utilized as reducing equivalent by all redox couples. Interestingly, there was significant increase in the activity of G6PD at 16 and 24 h (Figure 5G).

The Trx/TrxR system regulates protein thiol content in cell [65,66]. Oxidative modification of protein thiols and subsequent loss of function is one of the contributing factors for cell death after exposure to IR. Trx1 is associated with ASK1, once it is oxidized ASK1 is released from it and downstream signaling for apoptosis gets activated [67]. Trx is involved in reduction of disulfides on proteins critical for cell survival, DNA synthesis (ribonucleotide reductase) [68], repair, antioxidant defense (peroxiredoxin), cell proliferation (PTEN), and transcription factors (Nrf-2, NF-κB, AP1, P53, Hif1α, etc.) [17,69,70]. Trx provides reducing conditions inside nucleus for transcription factors to bind to DNA upon oxidative stress [50]. During early as well as late responses, there was a significant increase in activity of Trx and TrxR (Figure 6A–D). Intra- and inter-protein disulfides are formed upon induction of oxidative stress; increased activity of Trx system may help to maintain protein thiol pool in native state [71].

The above discussion clearly points out two important time points 16 and 24 h post IR exposure. At these time points, we observed cell cycle arrest (Figure 1B), increased concentration of total and intracellular thiols (Figure 3B and D), higher GSH/GSSG ratio (Figure 5B), increased activity of G6PD (Figure 5G), and increase in the activity of Trx and TrxR (Figure 6A–D). All these responses suggest that enhanced antioxidant buffering capacity of Jurkat cells may be responsible for their resistance to IR-induced apoptosis.

Thioredoxin (Trx) and glutathione system determine oxidative stress-tolerating capacity of tumor cells and hence they are the attractive targets for development of anticancer drugs [72]. ROS levels may be one of the contributing factors in determining tumor radio-resistance. When we monitored redox status of Trx- or GSH-deprived cells, we observed that these cells were harboring significantly higher basal ROS levels compared with control cells (Figure 7A and B). Addition of inhibitor of TrxR or GSH led to higher accumulation of ROS in Jurkat cells upon exposure to radiation indicating that these cells may exhibit radio sensitivity. Knocking down of TrxR1 or blocking GSH synthesis using an enzymatic inhibitor, BSO, did not induce significant cell death over control (Figure 7C and D). But addition of BSO to TrxR1 knockdown cells resulted in significant increase in cell death as compared with control. Knocking down of TrxR1 or blocking GSH synthesis before exposure to IR showed significant increase
Scheme 1. Antioxidant network regulates the cell fate after exposure to ionizing radiation

In conclusion, our results clearly demonstrate that although radiation generated significant levels of free radicals, tumor cells circumvent the toxic effects of ROS through upregulation of glutathione and Trx antioxidant networks leading to restoration of redox homeostasis. Thus, simultaneous disruption of glutathione or Trx metabolism enhanced radio-responsiveness of tumor cells which may result in beneficial outcome of radiotherapy.

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Declaration of interest
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**Supplementary material available online**

Supplementary Figure 1 to be found at online http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1056180.