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Ionizing radiation activates the Nrf2 antioxidant response

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Abstract

The transcription factor Nrf2 binds the antioxidant DNA response element (ARE) to activate important cellular cytoprotective defense systems. Recently several types of cancers have been shown to overexpress Nrf2, but its role in the cellular response to radiation therapy has yet to be fully determined. In this study, we report that single doses of ionizing radiation from 2-8Gy activate ARE-dependent transcription in breast cancer cells in a dose-dependent manner, but only after a delay of 5 days. Clinically relevant daily dose fractions of radiation also increased ARE-dependent transcription, but again only after 5 days. Downstream activation occurred of Nrf2-ARE-dependent gene and protein markers, such as heme oxygenase-1, whereas Nrf2-deficient fibroblasts were incapable of these responses. Compared to wild-type fibroblasts, Nrf2-deficient fibroblasts had relatively high basal levels of reactive oxygen species that increased greatly five days after radiation exposure. Further, in vitro clonogenic survival assays and in vivo sublethal whole body irradiation tests demonstrated that Nrf2 deletion increased radiation sensitivity, whereas Nrf2-inducing drugs did not increase radioresistance. Our results indicate that the Nrf2-ARE pathway is important to maintain resistance to irradiation, but that it operates as a second-tier antioxidant adaptive response system activated by radiation only under specific circumstances, including those that may be highly relevant to tumor response during standard clinical dose-fractionated radiation therapy.

Keywords

Ionizing Radiation; Nrf2; Antioxidant Response Element; Delayed Gene Expression; Heme Oxygenase-1

Introduction

Radiation therapy aims to eradicate clonogenic tumor cells and standard fractionated regimens exploit differences in the inherent radiation sensitivity of tumor cells relative to dose-limiting normal tissues to derive a radiotherapeutic benefit. Thus, a better understanding of the molecular determinants of tumor and normal tissue radiation sensitivity in radiation therapy is critical to improving clinical outcomes.

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Since ionizing radiation exerts its cytotoxicity in large part through the generation of reactive oxygen species (ROS), the natural antioxidant systems that cells use to maintain control of their redox balance are relevant for radiation therapy. One major system that reacts to oxidative stress to restore the redox balance involves genes coordinately regulated by transcription through the antioxidant response element (ARE). This is activated primarily by binding of the transcription factor NF-E2-related factor 2 (Nrf2), which belongs to a subset of basic leucine-zipper (bZip) genes with a conserved cap 'n' collar (CNC) domain. Under resting conditions, Nrf2 is sequestered in the cytoplasm by Keap1, an adaptor for a Cul3-based E3 ligase that promotes constitutive proteasome-mediated Nrf2 degradation (1,2). Under oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus where it upregulates ARE-dependent cytoprotective genes such as glutathione transferases (GSTs), UDP-glucuronosyltransferases, γ -glutamylcysteine synthetase (γ -GCS), glutathione peroxidase, heme oxygenase-1 (HO-1), catalase, and NAD(P)H:quinone oxidoreductase-1 (NQO-1). This adaptive response protects cells against acute and chronic endogenous and exogenous environmental stresses such as diesel exhaust particles, tobacco smoke, hyperoxia, inflammation, chemical carcinogens, and pulmonary and neurodegenerative diseases (3). Cancers, which are often in a continuous state of oxidative stress, appear to have their Nrf2-ARE pathway permanently activated resulting in enhanced tumor cell survival (4). By hijacking this pathway, tumor cells may also resist standard chemotherapy treatment (5).

Currently, we are aware of no reports showing that the Nrf2-ARE pathway impacts the outcome of radiation therapy, but this would seem likely since cytotoxicity from exposure to ionizing radiation is known to depend upon the levels of expression of numerous antioxidant enzymes and irradiation increases endogenous expression of many antioxidants, presumably by acting as an oxidative stress (6-9). We therefore hypothesized that radiation exposure would activate Nrf2 and that pre-activation of this pathway would be radioprotective. In this report, we used a stably expressing ARE-luciferase reporter cell line to monitor Nrf2 activity after exposure to ionizing radiation and tested if this Nrf2-dependent cytoprotective pathway would modulate intrinsic cell and tissue radiosensitivity. We found that basal levels of Nrf2-ARE activity affected intrinsic cellular radiosensitivity, as expected, but surprisingly irradiation activated this pathway only some time after exposure indicating that there is a hierarchy in the timing of antioxidant responses and that this major system makes a second tier response.

Materials and Methods

Animal Model

C57BL/6 Nrf2 heterozygous mice were a kind gift from Dr. Andre Nel (University of California, Los Angeles, CA) and were described previously (10). These animals and C3Hf/Kam mice were housed and bred in a defined-flora environment in the Association for the Assessment and Accreditation of Laboratory Animal Care International-accredited facilities of the Department of Radiation Oncology, University of California Los Angeles. The University of California at Los Angeles approved all experiments, which were done in accordance with all local and national guidelines for the care and use of animals.

Irradiation

C57BL/6 16-week old female Nrf2 WT, heterozygous, or KO mice were irradiated with 8Gy whole body irradiation from an AEC Cesium-137 source with a dose rate of 0.67Gy per minute. Body weight was measured every day for two weeks followed by every other day for an additional two weeks. Signs of radiation sickness were monitored and if overt morbidity was observed mice were euthanized in accordance to the approved protocol. Unless indicated otherwise, cells were irradiated using a Shepherd Cesium-137 irradiator with a dose rate of ~5Gy per minute.

Cell Cultures

Immortalized mouse embryonic fibroblast cells (MEFs) from Nrf2-deficient (KO) and wild-type (WT) mice were generously provided by Dr. Nobunao Wakabayashi (Johns Hopkins University, Baltimore, MD) (11) and were grown in Iscove's modified Dulbecco's medium (Invitrogen) with 10% FBS (Omega Scientific, Tarzana, CA). Confirmation of Nrf2 WT and KO status was tested by PCR using screening primers specific for WT Nrf2 or the lacZ insert (11). Murine dendritic cells (DC2.4) were a kind gift from Dr. Kenneth L. Rock (University of Massachusetts, MA) (12) and NIH-3T3 cells were obtained from ATCC (Manassas, VA). Cells were tested and confirmed by using cell surface markers with flow cytometry and identification of visual phenotypes. The MCF7-AREc32 cell line was kindly provided by Dr. Roland Wolf (University of Dundee, Scotland, UK) which stably expresses a luciferase gene construct controlled by 8 tandem copies of the ARE core sequence (13). MCF7-AREc32 cells were cultured in DMEM with 10% FBS containing 0.8 mg/mL of G418. Unless noted, cells were maintained in DMEM (Invitrogen) with 10% FBS (Omega Scientific, Tarzana, CA) and cultured at 37°C in humidified air containing 5% CO₂. Primary bone marrow cell for western blotting was harvested from the femurs by flushing with RPMI from WT mice 5 days after receiving 0 or 8Gy whole body irradiation. Each group contained four mice.

ARE-Luciferase Reporter Cell Assays

To test short term ARE activation by ionizing radiation, MCF7-AREc32 cells were plated at 1.2×10^4 cells per well in 96-well white plates in 100µL media, incubated overnight, irradiated and luciferase activity assessed over a 24 hour period. To monitor delayed activation of the ARE by ionizing radiation, cells were plated at 1×10^5 cells per dish in 10mm² dishes overnight, irradiated, harvested by trypsinization at the stated times, and plated in 100µL volumes in quadruplicate in a white 96-well plate. Luciferase activity was assessed following addition of 50µL of SteadyGlo reagent (Promega) in a SpectraMax M5 microplate reader (Molecular Devices). Data shown were normalized by cell number. Exogenous antioxidants glutathione (GSH), polyethylene glycol superoxide dismutase (PEG-SOD) and PEG-catalase (PEG-CAT) from Sigma were added after irradiation.

Clonogenic Assays

After treatment, cells were harvested and resuspended at the appropriate concentration in complete media. Following irradiation, they were plated in 10cm plastic Petri dishes in 10 mL of prewarmed media and incubated for 14 days. Colonies were fixed in 75% ethanol and stained with 0.5% crystal violet. The survival fraction was calculated as the number of colonies counted divided by the number of cells seeded times the plating efficiency at 0Gy. A linear-quadratic model was fitted to the data.

ROS Formation

To measure ROS formation, cells were incubated at 37°C in the dark with 20µM of 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFH-DA) or 5µM of dihydrorhodamine 123 (H₂DHR-123) for 30 minutes as adherent cells or in suspension. Adherent cells harvested via trypsinization and suspension cells were washed to remove the dye and were placed on ice prior to FACS analysis on a FACScalibur (Becton Dickinson, San Jose, CA) using CellQuest software with plots were generated by WinMDI 2.9.

Real-Time RT-PCR

Total RNA was extracted after treatment using TRIzol Reagent (Invitrogen) according to the manufactures instructions. Complimentary DNA (cDNA) synthesis was followed by quantitative PCR performed on an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). PCR conditions were set to 95°C for 3 minutes followed by 40 cycles of 95°C for 10

seconds, 60°C for 30 sec, and 72°C for 10 seconds. The C_t for each gene was determined after normalization to GAPDH, and $\Delta\Delta C_t$ was calculated relative to the designated reference sample. Gene expression values were then set equal to $2^{-\Delta\Delta C_t}$ as described by the iQ5 Optical System Software (Bio-Rad). All PCR primers were synthesized by Invitrogen and the sequences were as follows: GAPDH Forward 5'-AACTTTGGCATTGTGGAAGG-3' GAPDH Reverse 5'-ACACATTGGGGGTAGGAACA-3' HO-1 Forward 5'-TGGGTCCTCACTCTCAGCTT-3' HO-1 Reverse 5'-GTCGTGGTCAGTCAACATGG-3' GSTA-2 Forward 5'-GTATTATGTCCCCAGACCAAAGAG-3' GSTA-2 Reverse 5'-CTGTTGCCCACAAGGTAGTCTTGT-3'

Western Blotting

Cells were plated at 0.5×10^6 cells in 10 cm culture dishes and incubated overnight before treatment. After the stated treatments, cells were rinsed with ice-cold PBS and harvested with a cell scraper followed by centrifugation. The cell pellets were lysed for 10 minutes on ice followed by centrifugation at maximal speed. After protein quantification (Micro-BCA Protein Assay; Pierce, Rockford, IL), equal amounts of protein plus loading dye were added per lane on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel, electrophoresed, and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were blocked and probed with commercial antibodies for HO-1 (Stressgen), α -tubulin (CalbioChem) or GAPDH (Ambion) with a secondary anti-mouse or anti-rabbit HRP conjugated IgG antibody (GE Healthcare). Bands were visualized using ECL reagent (Pierce) on autoradiography film (Denville Scientific, Metuchen, NJ).

Results

Ionizing radiation does not activate ARE-reporter activity within 24 hours

The ARE-luciferase reporter cell line, MCF7-AREc32, was used to determine if exposure to varying doses of ionizing radiation would activate the Nrf2-ARE pathway. The radiation dose range was extended below that used in the clinic because low doses have been reported to increase antioxidant levels and to trigger adaptive radioresistance in human lymphocytes (6). In spite of this, no increase in ARE-reporter activity was observed after doses in the range 0.05 to 2Gy at varying times from 0.5 to 24 hours after exposure (Supplementary Fig. 1A and B). Even after doses of up to 10Gy, no activation was observed within a 24 hour period (data not shown). The Nrf2 inducer *tert*-butylhydroquinone (10 μ M tBHQ) was used to confirm that the reporter gene was functional, showing a dose- and time-dependent increase in reporter activity that by 24 hours was approximately 7-fold higher than in untreated cells (Supplementary Fig. 1C and data not shown).

Multiple fractions of ionizing radiation increase ARE-reporter activity

In the clinic, doses of radiation therapy are conventionally fractionated into a daily regimen. We therefore asked if fractionated delivery would activate the ARE. ARE-reporter cells were irradiated with 0.5, 1, 2, 3, or 4Gy every day for 5 days, harvested, and luciferase activity assayed 3 hours after the final dose. A dose dependent increase in luciferase expression was observed after 5 daily fractions from 0.5 to 4Gy (Fig. 1A). In contrast, 3 daily doses of 0.5, 2, or 4Gy did not significantly increase ARE activity at 24 hours after the final dose (data not shown).

Single doses of ionizing radiation activate ARE-reporter activity, but only days after exposure

Because ARE-reporter responses to fractionated radiation were delayed, the ability of single doses of between 2 and 8Gy to activate the ARE was investigated over an extended time period. Unlike after 24 hours, doses from 2 to 8Gy, clearly increased ARE-reporter activity when

assessed at 5 days (Fig. 1B). In all experiments corrections were made for radiation-induced cell loss and growth arrest at higher doses, although it was clear even before normalization by cell number that ionizing radiation activated the Nrf2-ARE pathway only late after exposure. In fact, the MCF7 cell line (14) displayed a dose-dependent cytostatic rather than a cytotoxic response to radiation rather than rapid interphase death within hours by apoptosis and like most cell lineages that exhibit mitotic cell death, autophagy, or senescence (15), and responded only over a period of many days. The ARE-reporter activity was found to persist past day 5 and was increasingly elevated when measured 8 and 15 days after a single dose of 4 and 8Gy. This activity subsided with continued passages in culture from days 21 to 31 post-irradiation (Fig. 1C). Furthermore, when continuously passaged cells were irradiated with 0.1 and 1Gy fractions 3 times per week every 24 hours for 3 weeks, a maximum of 7-fold change was noted in the 1Gy, but not the 0.1Gy group, again being maximal on day 15 (Supplementary Fig. 2). The ARE-reporter activity began to drop after day 15 regardless of receiving additional fractions.

Increased Nrf2-regulated gene and protein expression after cellular exposure to fractionated and single doses of radiation over time

To confirm that ARE-reporter activation was accurately reflected in downstream mRNA and protein production and that Nrf2 was driving these responses, wild type (WT) and Nrf2 knockout (KO) mouse embryo fibroblasts (MEFs) were irradiated daily with 0.5, 2, or 4Gy for five days and expression of mRNA for two ARE-driven genes HO-1 and GSTA2 assessed by real-time RT-PCR. Irradiation of WT MEFs gave dose-dependent increases in HO-1 and GSTA-2 expression while Nrf2 KO MEFs displayed no significant changes (Fig. 2A and B). Two additional cell lines, the fibroblast cell line NIH-3T3 (Fig. 2C) and the dendritic cell line DC2.4 (Fig. 2D), also responded with increased levels of HO-1 and GSTA2 mRNA. All these responses were delayed and single doses of 10Gy were ineffective at inducing expression at 24 hours (data not shown). To establish that fractionated irradiation might increase ARE expression *in vivo*, C57BL/6 mice were irradiated with 2Gy to the whole body every 24 hours for 5 days and the spleens harvested. HO-1 gene expression increased significantly after 5 daily doses of radiation, although GSTA2 mRNA levels remained low (Supplementary Fig. 3).

Western blotting for HO-1 expression confirmed that increased gene expression led to increased protein levels after five daily doses of 0.5, 2, or 4Gy in Nrf2 WT MEFs in a dose-dependent fashion, while in Nrf2 KO MEFs HO-1 protein was nearly undetectable (Fig. 3A). Primary WT MEFs responded in a similar manner as the immortalized WT MEFs (Fig. 3A), and single doses of radiation elicited similar HO-1 protein responses at 5 days in WT MEFs, primary WT MEFs, NIH-3T3 and primary bone marrow cells, but not Nrf2 KO MEFs (Fig. 3B). HO-1 expression in Nrf2 KO MEFs was increased only slightly by 8Gy radiation at 5 days, still not reaching basal WT levels.

Finally, function glutathione activity was found to be reduced by half in untreated Nrf2 KO MEFs compared to untreated WT and five days after exposure to 8Gy, was increased by ~50% in WT without a significant increase in the KO Nrf2 MEFs (Supplementary Fig. 4a). This trend remained true with 4Gy irradiation though was not found to have statistical significance.

Nrf2 activation before exposure is not radioprotective

To assess the radioprotective potential of the Nrf2-ARE pathway, several Nrf2-inducing agents were tested *in vitro* and *in vivo* for radiation modulating effects. First, the radiosensitive lymphocyte cell line, TIL-1, was pretreated 2, 6 or 16 hours with 0.01 to 5 μ M of the Nrf2-inducing agents sulforaphane, 2'-tert-butylhydroquinone (tBHQ), phenyl isocyanate (PEITC) none of which were found to significantly enhanced survival 24 hours after irradiation (data not shown). Second, a longer-term clonogenic survival assay using the apoptotic-resistant DC2.4 cell line, confirmed to have functional Nrf2 activity, was pre-treated with 10 μ M tBHQ

for 1 to 24 hours before irradiation but this did not alter clonogenic survival (data not shown). Finally, C3H mice were treated 24 hours and 1 hour before lethal whole body irradiation with subcutaneous injections of 60mg/kg of tBHQ (n=8) or 10mg/kg of PEITC (n=8) neither of which were significantly protective compared to vehicle treated controls that were euthanized from days 12 to 14 due to radiation sickness (n=16). These results suggest that activation of the Nrf2-ARE pathway under normal steady state conditions does not affect cell or animal survival in response to subsequent irradiation.

Loss of Nrf2 increases intrinsic cellular radiosensitivity

While Nrf2 activation prior to exposure to ionizing radiation offered no significant radiation protection under steady state conditions, we sought to determine the impact of the basal levels of Nrf2 activity on intrinsic cellular radiosensitivity. Clonogenic assays were performed with WT and Nrf2 KO MEFs. Loss of Nrf2 rendered the MEFs significantly more radiosensitive (Fig. 4C). We found no measureable difference in the cell cycle parameters for Nrf2 WT and KO MEFs at the time of irradiation that could have had an influence on radiation sensitivity (data not shown). At 10% survival, the radiation protection factor was 1.3, supporting an intrinsic radioprotective role for the basal Nrf2-ARE pathway.

To test the effects of loss of Nrf2 on radiosensitivity *in vivo*, age-matched, female Nrf2 WT and heterozygous and homozygous Nrf2-deficient mice on a C57BL/6 background were treated with 8Gy whole body irradiation. This is a sublethal dose for our C57BL/6 mice, that have a LD50/30 of 8.75Gy (50% death within 30 days). Severe morbidity in 3 of 3 KO mice necessitated their euthanasia at days 9, 13 and 16 post-irradiation. These mice displayed classical signs of radiation-induced sickness including weight loss, rough coat, difficulty breathing, kyphosis, and decreased mobility. Nrf2 WT (n=3) and heterozygous (n=2) littermate control mice irradiated simultaneously displayed only slight weight loss within the first week, but recovered and showed no other visual signs of morbidity. Thus, loss of the Nrf2-ARE pathway *in vitro* and *in vivo* enhanced radiosensitivity.

ROS formation is increased by irradiation in Nrf2-deficient cells

From the above data, we concluded that Nrf2 pathways control basal redox levels and hypothesized that this is sufficient to minimize many effects of radiation-induced ROS. We sought to explore this further in WT and Nrf2 KO MEFs using cell-permeable ROS-sensitive fluorescent dyes. Cells were incubated with the two ROS probes 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFH-DA) or dihydrorhodamine 123 (H₂DHR-123) prior to irradiation and FACS analysis (16-18). The former is a useful indicator of general oxidative stress due to its broad reactivity with a variety of ROS, while the latter is more specific for ROS generated via hydrogen peroxide. Nrf2 KO MEFs had higher fluorescence levels than WT MEFs with both dyes (Fig. 4A and B), indicating that under basal conditions, Nrf2 KO MEFs have a lower capacity to maintain a redox buffering system. The ROS response to irradiation with 0, 2, 4, 8, or 10Gy was examined using cells loaded with H₂DCFH-DA. WT cells showed a marginal 2-fold increase in ROS after 8 and 10Gy, but not lower doses, whereas irradiation dramatically enhanced ROS formation in irradiated KO MEFs (>10-fold after 8Gy) (Fig. 5).

Delayed oxidative stress after exposure to ionizing radiation

Since radiation-induced ARE activation showed a 5 day time delay, ROS formation was examined over the same time scale. WT and Nrf2 KO MEFs were treated with 8Gy, incubated for 1 or 5 days, stained with 20μM of H₂DCFH-DA, washed and analyzed by FACS (Fig. 6). Although Nrf2 KO MEFs had higher basal ROS levels, irradiation did not increase these at 24 hours. However, by day 5 ROS formation was increased 2.5-fold in the irradiated WT and almost 6-fold in the KO MEFs (Fig. 6C). Furthermore, the addition of exogenous antioxidants

glutathione and PEG-SOD but not PEG-CAT after irradiation partially decreased ARE-reporter activity after five days (Supplementary Fig. 4b-d). These results are consistent with delayed generation of ROS after irradiation and the role of Nrf2 activation in responding to this challenge.

Discussion

These data demonstrate that ionizing radiation can activate Nrf2 to increase ARE-dependent gene expression, but only after a significant time delay. In the stably expressing luciferase reporter cell line, MCF7-AREc32, the ARE was responsive only 5 days after daily fractionated or a single larger radiation dose. These responses were obvious with or without correction for radiation-induced cell death.

Irradiation is known to drive expression of gene transcriptional programs, such as through AP-1 or NF- κ B activation, that can peak within 24 hours (19) and that are under redox control. In contrast, antioxidants such as mitochondrial MnSOD can be induced within 24 hours after irradiation in particular by NF- κ B pathway activation. Human lymphoblasts exposed to 3Gy had higher levels of catalase and glutathione peroxidase, but not other antioxidants, at 20 hours and an elevated early expression of a range of enzymes after a conditioning dose of 0.02Gy that suggested a low-dose adaptive antioxidant, radioprotective response (6). Expression of glutathione S-transferase Pi, NADH dehydrogenase, and peroxiredoxin VI also were found to be upregulated within 24 hours in livers, but not in the spleen, of mice receiving whole body irradiation (7) indicating some selectivity in the response. Very recently, a report by Tsukimoto *et al.* demonstrated low doses of 0.1 to 2.5Gy gamma-radiation induced nuclear accumulation of Nrf2 by 4 hours and elevated HO-1 expression at 24 hours in the mouse macrophage cell line Raw 264.7 (20). Since we were unable to observe enhanced ARE-reporter activity and HO-1 expression at 24 hours after irradiation in a variety of cell types, early activation of the Nrf2-ARE pathway may be cell type dependent. In contrast, we found Nrf2-ARE reporter gene activity only 5 days after exposure and HO-1 mRNA and/or protein expression to be elevated with a delayed time course in primary and immortalized non-tumorigenic WT MEFs, the established fibroblast cell line NIH-3T3, dendritic cell line DC2.4, as well as in primary bone marrow cells. An increase in functional activity of glutathione was also demonstrated at day 5 in Nrf2 WT MEFs but not KO.

This highly robust Nrf2-ARE mediated antioxidant response that took 5 days to express itself was radiation dose- as well as time-dependent and correlate with delayed ROS production as measured by fluorescent ROS-sensitive dyes. Interestingly, drugs that rapidly induced Nrf2 did not affect cellular radiation responses but loss of Nrf2 rendered cells and mice radiosensitive, and this also was associated with higher basal and radiation-induced ROS levels. This suggests that the steady state Nrf2 pathway products buffers redox changes induced by irradiation adequately and that higher levels are not required, but that ROS levels continue to climb 5 days and by 5 days are sufficient to induce a second tier antioxidant response, the first being early induction of mitochondrial MnSOD and related enzymes. This is in keeping with the data of Haton *et al.* who found that radiation induced MnSOD and thioredoxin 2 expression in the gut by 6 hours after abdominal exposure, but by 4 days the response had waned and a second level of induced anti-oxidant genes were expressed including glutathione peroxidases and metallothioneins (21). These antioxidants systems coordinate responses. Pardo and colleagues found that overexpression of MnSOD stabilized the antioxidant pool including glutathione and total thiols leading to production of heme oxygenase-1, glutamate-cysteine ligase, and NF-E2-related factor-2 (Nrf2) (22). On the other hand Nrf2 can regulate MnSOD production, as well as glutathione transferases, heme oxygenase-1 (HO-1), and NAD(P) H:quinone oxidoreductase-1 (NQO-1) (23-25). The addition of exogenous antioxidants glutathione and PEG-SOD partially decreased ARE-reporter activity at day 5 after 8Gy but

PEG-CAT had no effect suggesting the source of oxidative stress may determine activation. Determination of how these various scavengers interrelate in a time- and dose-dependent fashion may provide insight into unraveling the aspects of cellular recovery.

This study raises a major question with respect to delayed effects of ionizing radiation. Radiation-induced ROS generated by extranuclear amplification mechanisms, in which mitochondria play a prominent role, may promote delayed radiation-induced pro-oxidant cycles of production that disturbs the balance of intracellular metabolic pro-oxidation/anti-oxidant (redox) reactions long-term (26). In fact there is evidence that redox disturbances and high ROS levels can persist through many cell generations, are associated with mitochondrial dysfunction, and may contribute to genomic instability (27). There are several reports of oxidative stress persisting days to weeks after exposure to ionizing radiation and the suggestion made that this can lead to chromosomal instability (28-31). Persistent oxidative stress was first observed 7 days after 1Gy irradiation in mature bone marrow cultures (32), findings that have been repeated in several independent studies in other cell culture systems and shown to be attenuated by addition of free radical scavengers (30,33). Similarly, radiation-induced chromosomal instability can occur in a delayed fashion in the progeny of irradiated cells (34) being observed in the bone marrow as long as 24 months after whole body irradiation of mice with x-rays or neutrons (29,35). Our studies do not speak to chromosomal instability, but do indicate that ROS are generated long after radiation exposure is over. The mechanism responsible for delayed ROS production is unknown but is most likely related to waves of cell death or pro-inflammatory cytokine production, both of which are known to be produced in a cyclical fashion over days, weeks, and months after irradiation (36,37) and both of which can generate ROS (38). Within this context, the prime role of Nrf2-related pathways would be in control of cellular homeostasis, host inflammation and immunity. In fact, Nrf2 activation plays a key role in mitigating runaway inflammation during septic shock (39).

The basal level of ROS depends on the steady state level of antioxidants in the cell and while these may be adequate to buffer radiation-induced redox fluctuations in many cells under normal conditions, this was not the case for Nrf2 KO MEFs, where ROS levels increased immediately following irradiation while WT MEFs had lower basal ROS levels that were unchanged following radiation exposure. Furthermore, it seems that the level of control that Nrf2 maintains over redox generally affects cell survival and preserves tissue function. In clonogenic assays, loss of Nrf2 increased intrinsic cellular radiation sensitivity, and Nrf2 KO mice were more sensitive to whole body sub-lethal irradiation compared to wild-type and heterozygous littermates. This finding has recently been confirmed using an RNAi-based model system in non-small cell lung cancers showing reduced survival in clonogenic assays by downregulation of Nrf2 and furthermore enhanced radiation resistance in genetically engineered MEF cells with constitutively activated Nrf2 (40,41). Also, increased Nrf2 expression has been detected by immunohistochemistry in hamster oral mucosa only at day 19 after a fractionated regime of 8×7.5Gy (42). This coincided with a peak in mucositis and the beginning of the healing process. The role of Nrf2 in wound healing has been previously examined using full-thickness excisional wounds of the dorsal midline in Nrf2 WT and KO mice, in part because Nrf2 was identified as a transcriptional target for keratinocyte growth factor (43,44). Pro-inflammatory cytokine levels in wounds were actually decreased in the Nrf2 KO mice but there was prolonged inflammation in the late phase of repair (43), even though wound healing did not appear to be affected presumably because Nrf3 or other pathways contributed. The wound healing process as it evolves from inflammation to tissue repair and remodeling requires that a pro-oxidant, pro-inflammatory phase transition into an antioxidant, anti-inflammatory phase tissue repair phase and the Nrf2 pathway may participate in this process, after an appropriate delay. The report that Nrf2 attenuates NF- κ B signaling (45) is consistent with this concept.

The Nrf2-ARE pathway has therefore several possible roles in response to irradiation. By determining the basal redox status in a cell, it may modulate intrinsic cellular radiosensitivity. The dysregulation of Nrf2 in cancer has been suggested to protect and offer growth advantages to various cancers (46-48) and may offer resistance to chemotherapy (5). Whether or not interventions targeted at this pathway will be useful in radioprotection will depend upon the buffering capacity that exists within the system, but extensive downregulation may radiosensitize cancers for more effective treatment. Perhaps more importantly, by down-regulating radiation-induced inflammation, including persisting responses that can be associated with radiation-induced late normal tissue injury (49), Nrf2 may encourage tissue repair and functional recovery and at the same time decrease the likelihood of radiation-induced cancers. This role deserves additional attention as manipulation of this pathway may provide key benefits in mitigating late normal tissue complications arising from clinical radiotherapy or other radiation exposures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

MCF7-AREc32 reporter activity measured (A) 3 hours after five daily fractions of irradiation, (B) 5 days after single doses of radiation or (C) multiple days after a single dose of 4 or 8Gy that were passaged on days 8, 15, 21 and 26. Data was normalized to the control with equal numbers of viable cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different compared to control.

Figure 2.

Repeated fractions of radiation enhanced mRNA expression of HO-1 and GSTA-2 genes. Daily irradiation of 0.5, 2, or 4Gy for five days enhanced expression of (A.) HO-1 and (B.) GSTA-2 mRNA in WT but not Nrf2 deficient MEFs as determined by real-time RT-PCR 3 hours after the last radiation dose. Similar results are obtained in two additional cell lines (C.) NIH-3T3 fibroblast cells and (D.) the dendritic cell line DC2.4. Data are normalized to the unirradiated WT control. The average of two independent experiments is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different compared to control.

Figure 3. Western blot of HO-1 expression after irradiation. (A.) 5 daily fractions of radiation in WT or Nrf2 KO MEFs and primary WT MEFs. (B.) 5 days after single doses of radiation to WT or Nrf2 KO MEFs, primary WT MEFs, NIH-3T3 fibroblast cells and primary bone marrow cells (BMC). Top row: HO-1, bottom row: GAPDH.

Figure 4.

Basal ROS formation and intrinsic radiosensitivity of WT and Nrf2 KO MEFs. Adherent cells were loaded with (A.) 20 μ M of H₂DCFH-DA or (B.) 5 μ M of H₂DHR-123 in PBS for 30 minutes, harvested and fluorescence was measured by FACS. (C) Survival assay of WT or Nrf2-deficient MEFs in irradiated in single cell suspensions and plated for clonogenic survival.

Figure 5.

FACS analysis of ROS formation using the H₂DCFH-DA probe in WT or Nrf2 deficient MEFs after exposure to ionizing radiation. Cells were loaded with H₂DCFH-DA for 30 minutes, irradiated, placed on ice and analyzed via FACS. Representative data for 0 and 10Gy with (A.) Nrf2 WT and (B.) Nrf2 KO MEFs are shown. (C.) Mean fluorescence from three independent experiments with varying radiation doses. *p<0.05, significant difference between Nrf2 WT compared to Nrf2 KO.

Figure 6. FACS analysis of ROS formation in (A.) WT and (B.) Nrf2 KO MEFs using the H₂DCFH-DA probe at 1 day and 5 days after exposure to 8Gy. (C.) Mean fluorescence from three independent experiments in WT and KO MEFs 5 days after treatment with 0 or 8Gy. *p<0.05, significantly different compared to wild-type.