Ionizing Radiation Activates the Nrf2 Antioxidant Response


Abstract

The transcription factor NF-E2-related factor 2 (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 to 8 Gy activate ARE-dependent transcription in breast cancer cells in a dose-dependent manner, but only after a delay of five days. Clinically relevant daily dose fractions of radiation also increased ARE-dependent transcription, but again only after five days. Downstream activation of Nrf2-ARE-dependent gene and protein markers, such as heme oxygenase-1, occurred, whereas Nrf2-deficient fibroblasts were incapable of these responses. Compared with 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 showed 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. Cancer Res; 70(21); 8886–95. © 2010 AACR.

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.

Because 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 genes with a conserved cap “n” collar 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). Further, in vitro clonogenic survival assays and in vivo sublethal whole body irradiation tests showed 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.
many antioxidants, presumably by acting as an oxidative stress (6–9). We therefore hypothesized that radiation exposure would activate Nrf2 and that preactivation 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 cytotoxic 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 at the facilities of the Department of Radiation Oncology, University of California Los Angeles, which are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. 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

Sixteen-week-old female C57BL/6 Nrf2 wild-type (WT), heterozygous, or deficient (KO) mice were irradiated with 8 Gy whole-body irradiation from an AEC Cesium-137 source with a dose rate of 0.67 Gy/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 with the approved protocol. Unless indicated otherwise, cells were irradiated using a Shepherd Cesium-137 irradiator with a dose rate of ~5 Gy/minute.

Cell cultures

Immortalized mouse embryonic fibroblast cells (MEF) from Nrf2 KO and WT mice were generously provided by Dr. Nobuao Wakabayashi (Johns Hopkins University, Baltimore, MD; ref. 11) and were grown in Iscove’s modified Dulbecco’s medium (Invitrogen) with 10% fetal bovine serum (Omega Scientific) 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 five days after receiving 0 or 8 Gy 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 × 104 cells per well in 96-well white plates in 100 μL media, incubated overnight, and irradiated, and luciferase activity was assessed over a 24-hour period. To monitor delayed activation of the ARE by ionizing radiation, cells were plated at 1 × 105 cells per dish in 10-mm2 dishes overnight, irradiated, harvested by trypsinization at the stated times, and plated in 100 μL volumes in quadruplicate in a white 96-well plate. Luciferase activity was assessed following addition of 50 μL 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 10-cm 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 0 Gy. 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 20 μmol/L of 2’7’-dichlorodihydrofluorescein diacetate (H2DCFH-DA) or 5 μmol/L of dihydrohodamine 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 plated in 10-mm2 dishes overnight, irradiated, harvested by trypsinization at the stated times, and plated in 100 μL volumes in quadruplicate in a white 96-well plate. Luciferase activity was assessed following addition of 50 μL 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.

Real-time reverse transcriptase-PCR

Total RNA was extracted after treatment using TRizol Reagent (Invitrogen) according to the manufacturer’s instructions. cDNA synthesis was followed by quantitative PCR performed on an iQ5 Real-Time PCR Detection System.
PCR conditions were set to 95°C for 3 minutes followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, 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′-GTAT-TATGTCCCCCAGACCAAAGAG-3′; GSTA-2 reverse 5′-CTGTTGCCCAAGGTTAGTCTTGT-3′.

Western blotting
Cells were plated at 0.5 × 10⁶ 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), equal amounts of protein plus loading dye were added per lane on a 12% SDS-polyacrylamide gel, electrophoresed, and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked and probed with commercial antibodies for HO-1 (Stressgen), α-tubulin (CalbioChem) or GAPDH (Ambion) with a secondary antimouse or antirabbit horseradish peroxidase–conjugated IgG antibody (GE Healthcare). Bands were visualized using ECL reagent (Pierce) on autoradiography film (Denville Scientific).

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 2 Gy at varying times from 0.5 to 24 hours after exposure (Supplementary Fig. S1A and B). Even after doses of up to 10 Gy, no activation was observed within a 24-hour period.
The Nrf2 inducer tert-butylhydroquinone (10 μmol/L 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. S1C 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 4 Gy every day for five days, harvested, and luciferase activity assayed three hours after the final dose. A dose-dependent increase in luciferase expression was observed after five daily fractions from 0.5 to 4 Gy (Fig. 1A). In contrast, three daily doses of 0.5, 2, or 4 Gy 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 8 Gy to activate the ARE was investigated over an extended time period. Unlike after 24 hours, doses from 2 to 8 Gy 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 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 8 Gy. This activity subsided with continued passages in culture from days 21 to 31 postirradiation (Fig. 1C). Furthermore, when continuously passaged cells were irradiated with 0.1 and 1 Gy fractions 3 times per week every 24 hours for 3 weeks, a maximum of 7-fold change was noted in the 1 Gy, but not the 0.1 Gy group, again being maximal on day 15 (Supplementary Fig. S2). The ARE-reporter activity began to drop after day 15 regardless of receiving additional fractions.

**Figure 2.** Repeated fractions of radiation-enhanced mRNA expression of HO-1 and GSTA-2 genes. Daily irradiation of 0.5, 2, or 4 Gy for five days enhanced expression of HO-1 (A) and GSTA-2 (B) mRNA in WT but not Nrf2 KO MEFs as determined by real-time reverse transcriptase-PCR 3 hours after the last radiation dose. Similar results are obtained in two additional cell lines: NIH-3T3 fibroblast cells (C) and the dendritic cell line DC2.4 (D). 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 with control.
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, WT and Nrf2 KO mouse MEFs were irradiated daily with 0.5, 2, or 4 Gy for five days, and expression of mRNA for two ARE-driven genes, HO-1 and GSTA2, was assessed by real-time reverse transcriptase-PCR. Irradiation of WT MEFs gave dose-dependent increases in HO-1 and GSTA2 expression whereas 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 10 Gy 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 2 Gy to the whole body every 24 hours for 5 days and the spleens were harvested. HO-1 gene expression increased significantly after five daily doses of radiation, although GSTA2 mRNA levels remained low (Supplementary Fig. S3).

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 4 Gy in Nrf2 WT MEFs in a dose-dependent fashion, whereas 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 five days in WT MEFs, primary WT MEFs, NIH-3T3, and primary bone marrow cells, but not in Nrf2 KO MEFs (Fig. 3B). HO-1 expression in Nrf2 KO MEFs was increased only slightly by 8 Gy radiation at five days, still not reaching basal WT levels.

Finally, function glutathione activity was found to be reduced by half in untreated Nrf2 KO MEFs compared with untreated WT, and five days after exposure to 8 Gy was increased by ~50% in WT without a significant increase in the KO Nrf2 MEFs (Supplementary Fig. S4A). This trend remained true with 4 Gy irradiation but 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 μmol/L of the Nrf2-inducing agents sulforaphane, 2'-tert-butylhydroquinone (tBHQ), and phenyl isocyanate (PEITC), none of which was found to significantly enhance survival 24 hours after irradiation (data not shown). Second, in a longer-term clonogenic survival assay the apoptotic-resistant DC2.4 cell line, confirmed to have functional Nrf2 activity, was pretreated with 10 μmol/L 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 s.c. injections of 60 mg/kg tBHQ (n = 8) or 10 mg/kg PEITC (n = 8), neither of which were significantly protective compared with 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

Although 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 measurable 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 8 Gy whole-body irradiation. This is a sublethal dose for our C57BL/6 mice, which have a LD50/30 (50% death within 30 days) of 8.75 Gy. Severe morbidity in 3 of 3 KO mice necessitated their euthanasia at days 9, 13, and 16 postirradiation. 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.

**Figure 4.** Basal ROS formation and intrinsic radiosensitivity of WT and Nrf2 KO MEFs. Adherent cells were loaded with 20 μmol/L of H2DCFH-DA (A) or 5 μmol/L of H2DHR-123 (B) in PBS for 30 minutes, harvested, and fluorescence was measured by FACS. C, survival assay of WT or Nrf2 KO MEFs 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 KO 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 10 Gy with Nrf2 WT (A) and Nrf2 KO (B) MEFs are shown. C, mean fluorescence from three independent experiments with varying radiation doses. *, *P* < 0.05, significant difference between Nrf2 WT compared with Nrf2 KO.

**ROS formation is increased by irradiation in Nrf2-deficient cells**

From the above data, we concluded that Nrf2 pathways control basal redox levels, and we hypothesized that this is sufficient to minimize many effects of radiation-induced ROS. We sought to explore this farther in WT and Nrf2 KO MEFs using cell-permeable ROS-sensitive fluorescent dyes. Cells were incubated with the two ROS probes, H2DCFH-DA or 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, whereas 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 10 Gy was examined using cells loaded with H$_2$DCFH-DA. WT cells showed a marginal 2-fold increase in ROS after 8 and 10 Gy, but not lower doses, whereas irradiation dramatically enhanced ROS formation in irradiated KO MEFs (>10-fold after 8 Gy; Fig. 5).

**Delayed oxidative stress after exposure to ionizing radiation**

Because 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 8 Gy, incubated for 1 or 5 days, stained with 20 μmol/L of H$_2$DCFH-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. By day 5, however, 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. S4B–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 show 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 five 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-κB 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-κB pathway activation. Human lymphoblasts exposed to 3 Gy 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 0.02 Gy that suggested a low-dose adaptive antioxidant, radioprotective response (6). Expression of glutathione S-transferase Pi, NADH dehydrogenase, and peroxiredoxin VI was also found to be upregulated within 24 hours in the liver, 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. showed that low doses of 0.1 to 2.5 Gy γ-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). Because 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 five days after exposure and HO-1 mRNA and/or protein expression to be elevated with a delayed time course in primary and immortalized nontumorigenic WT MEFs, the established fibroblast cell line NIH-3T3, dendritic cell line DC2.4, and in primary bone marrow cells. An increase in functional activity of glutathione was also shown at day 5 in Nrf2 WT MEFs but not in KO.

This highly robust Nrf2-ARE-mediated antioxidant response that took five days to express itself was radiation dose and time dependent, and correlates 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 product buffers redox...
changes induced by irradiation adequately and that higher levels are not required, but that ROS levels continue to climb for five days and by five 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 six hours after abdominal exposure, but by four days the response had waned and a second level of induced antioxidant genes was expressed, including glutathione peroxidases and metallothioneins (21). These antioxidant 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 Nrf2 (22). On the other hand, Nrf2 can regulate MnSOD production, as well as glutathione transferases, HO-1, and NQO-1 (23–25). The addition of exogenous antioxidants glutathione and PEG-SOD partially decreased ARE-reporter activity at day 5 after 8 Gy, 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 disturb the balance of intracellular metabolic pro-oxygenation/antioxidant (redox) reactions in the 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 for days to weeks after exposure to ionizing radiation, and it has been suggested that this can lead to chromosomal instability (28–31). Persistent oxidative stress was first observed seven days after 1 Gy 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 proinflammatory 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 although 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 whereas 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 sublethal irradiation compared with wild-type and heterozygous littermates. This finding has recently been confirmed using an RNA interference–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). Moreover, increased Nrf2 expression has been detected by immunohistochemistry in hamster oral mucosa only at day 19 after a fractionated regime of 8 × 7.5 Gy (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). Proinflammatory cytokine levels in wounds were actually decreased in the Nrf2 KO mice but there was prolonged inflammation in the late phase of repair (43), although wound healing did not seem 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 the pro-oxidant, proinflammatory 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-κB 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 downregulating 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.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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