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SOD2-mediated Adaptive Responses Induced by Low Dose Ionizing Radiation via TNF Signaling and Amifostine

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Abstract

Manganese superoxide dismutase (SOD2)-mediated adaptive processes that protect against radiation-induced micronuclei formation can be induced in cells following a 2 Gy exposure by previously exposing them to either low dose ionizing radiation (10 cGy) or WR1065 (40 μ M), the active thiol form of amifostine. While both adaptive processes culminate with elevated levels of SOD2 enzymatic activities, the underlying pathways differ in complexity, with the tumor necrosis factor α (TNF α) signaling pathway implicated in the low dose radiation-induced response, but not in the thiol-induced pathway. The goal of this study was the characterization of the effects of TNF α receptors 1 and 2 (TNFR1, 2) on the adaptive responses induced by low dose irradiation or thiol exposures using micronuclei formation as an endpoint. BFS-1 wild type (WT) cells with functional TNFR1 and 2 were exposed 24 h prior to a 2 Gy dose of ionizing radiation to either 10 cGy or a 40 μ M dose of WR1065. BFS2C-SH02 cells defective in TNFR1 and BFS2C-SH22 cells defective in both TNFR1 and 2, generated from BFS2C-SH02 cells by transfection with a murine TNFR2 targeting vector and confirmed to be TNFR2 defective by quantitative PCR, were also exposed under similar conditions for comparison. A 10 cGy dose of radiation induced a significant elevation of SOD2 activity in BFS-1 ($P < 0.001$) and BFS2C-SH02 ($P = 0.005$) but not BFS2C-SH22 cells ($P = 0.433$) as compared to their respective untreated controls. In contrast, WR1065 significantly induced elevations in SOD2 activity in all three cell lines ($P = 0.001$; $P = 0.007$; $P = 0.020$; respectively). A significant reduction in the frequency of radiation-induced micronuclei was observed in each cell line when exposure to a 2 Gy challenge dose of radiation occurred during the period of maximal elevation in SOD2 activity. However, this adaptive effect was completely inhibited if the cells were transfected 24 h prior to low dose radiation or thiol exposure with SOD2 siRNA. Under the conditions tested, TNFR1,2 inhibition negatively impacted the low dose radiation-induced but not the thiol-induced adaptive responses observed to be mediated by elevations in SOD2 activity.

Introduction

Exposure of cells to low dose ionizing radiation has been reported to induce an elevated resistance in cells subsequently exposed to a much higher dose of radiation. This phenomenon, first reported in 1984, has been identified as a radio-adaptive response

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characterized by either an elevation in cell survival and/or a reduction in genomic damage as measured by markers of genomic instability [1–3]. While this phenomenon has not been universally observed in all cell systems or even in similar cell types from different individuals, it has been demonstrated to occur in sufficient frequency in cultured cells, as well as rodent and human cell systems to be identified as an important consequence of low dose radiation exposure.

Numerous investigations have been performed to characterize the adaptive response and to identify the underlying molecular pathways responsible for its expression. One such pathway that appears to be associated with a radio-induced adaptive response involves the nuclear factor κ B (NF κ B) signaling pathway that leads to an elevated expression of the manganese superoxide dismutase (*SOD2*) gene and a subsequent elevation of SOD2 enzymatic activity [4–6]. An important component of this pathway is an intronic NF κ B element in the *SOD2* gene that has been reported to be essential for the rapid activation of the gene following exposure to stress-inducing agents such as ionizing radiation and tumor necrosis factor- α (TNF α) [7]. The TNF signaling pathway has also been identified as being an important component of a radiation-induced adaptive response [8–10]. TNF is a pleiotropic cytokine that is induced by ionizing radiation and can exert its effects via two separate receptors, TNFR1 having a molecular mass of 55 kd and TNFR2 having a molecular mass of 75 kd [11]. These receptors are expressed on most cell types and initiate signaling through the recruitment of cytosolic proteins through protein-protein interaction domains in their cytoplasmic regions [12]. While both TNFR1 and TNFR2 possess sequences that are capable of binding to various adaptor proteins capable of eliciting both similar and different signaling pathways, NF κ B activation appears to be a common target for activation through both receptor driven pathways [13–16]. Activation of NF κ B can then result in a subsequent elevation of *SOD2* gene expression [17]. The elevation of SOD2 enzymatic activity induced in this manner reaches maximal levels between 20 and 24 h later, at which time if cells are challenged with a second high dose of ionizing radiation their relative resistance to cell killing can increase by about 40% [17]. The importance of both NF κ B signaling and elevated SOD2 activity in the expression of this radio-induced adaptive response has been further demonstrated through the use of NF κ B inhibitors [18, 19] and/or *SOD2* siRNA transfection of cells [20, 21], both of which result in the abolishment of elevated SOD2 activity and concomitant enhanced cell survival or reduced micronuclei formation, a marker of genomic instability.

An analogous phenomenon to the radiation-induced adaptive response involving the activation of *SOD2* gene expression and elevation of SOD2 activity in cells has been observed following exposure of cells to thiol-containing reducing drugs that include *N*-acetylcysteine (NAC), amifostine, mesna, oltipraz, and captopril [22–24]. A mechanism of action identified for the induction of this effect by thiols involves the altering of the redox state of cysteine residues in the p50 and p65 subunits of NF κ B resulting in NF κ B activation and a subsequent elevation of *SOD2* gene expression [24, 25]. As in the case of the radiation-induced adaptive response, periods of elevated SOD2 activity induced by thiols correlated with enhanced resistance to cell killing and genomic instability induced by exposure to high doses of ionizing radiation, and these effects could be abolished through the use of inhibitors of NF κ B and/or *SOD2* siRNA transfection [17–21, 24, 26, 27]. The similarity of the responses associated with both the radiation- and thiol-induced “adaptive” responses suggests that they may share a common molecular pathway. To investigate this possibility, we assessed the existence and magnitude of radiation- and thiol-induced adaptive responses in the wild type BFS-1 and the TNFR1 knockout BFS2C-SH02 (TNFR1[−]) and TNFR1,2 knockout BFS2C-SH22 (TNFR1[−]R2[−]) fibrosarcoma cell lines. Since both of the TNF receptors are implicated in the activation of NF κ B signaling, it was important to use cells defective in both receptors, TNFR1[−]R2[−], to insure a complete

disruption of the TNF signaling pathway. In particular, earlier work with a RKO human colon carcinoma cell line suggested that the magnitude and kinetics of SOD2 induction by TNF α were similar to that observed following exposure of cells to the free thiol form of amifostine, e.g., WR1065 [17]. We now investigate the role of the TNF signaling pathway in the thiol as compared to radiation-induced adaptive response.

Materials and Methods

Cells and culture conditions

Wild type BFS-1 cells were obtained from a 3-methylcholanthrene-induced fibrosarcoma generated in a wild type female C57BL/6 mouse. Tumor necrosis factor receptor 1 (TNFR1)-deficient cells were obtained from a 3-methylcholanthrene-induced tumor generated in a female TNFRp55 $^{-/-}$ deficient mouse and were kindly provided by Dr. D.N. Männel, Institute of Pathology/Tumor Immunology, University of Regensburg, Regensburg, Germany [11]. Wild type BFS-1 cells adapted for *in vitro* growth and TNFR1 knockout cells designated BFS2C-SH02 were maintained in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), penicillin (100 units/ml) and streptomycin (100 mg/ml) (Invitrogen Life Technologies) at 37 °C in 5% CO $_2$ and 95% air. TNFR1 $^{-}$ R2 $^{-}$ cells were generated by transfecting BFS2C-SH02 cells with 2 μ g TRCN0000012322 (murine TNFR2 targeting; Sigma, St. Louis, MO, USA) plasmid DNA using Fugene HD (Roche, Indianapolis, IN, USA). Stable transfectants were selected using 1 μ g/ml puromycin. Expression of TNFR2 in the various transfected clones was confirmed by quantitative PCR, and the greatest reduction was found to be 50% of the BFS2C-SH02 levels. This clone was designated BFS2C-SH22 (TNFR2 targeted). All experiments were performed using BFS-1 WT, BFS2C-SH02 (TNFR1 $^{-}$) and BFS2C-SH22 (TNFR1 $^{-}$ R2 $^{-}$) cells grown to confluence and then refed with fresh medium and maintained for an additional 3 days. Cultures were again refed with fresh medium 1 day prior to each experiment.

Irradiation conditions

BFS-1, BFS2C-SH02 and BFS2C-SH22 cells, normal control or transfected with *SOD2* siRNA, were irradiated at room temperature using a Phillips X-ray generator operating at 250 kVp and 15 mA at a dose rate of 0.368 Gy/min to deliver a dose of 10 cGy, or 1.65 Gy/min to deliver a dose of 2 Gy.

Treatment with WR1065, the active free thiol form of amifostine

WR1065 (2-[(aminopropyl)amino]ethanethiol), the active thiol metabolite of amifostine, was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Immediately before use, the drug was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 M and was then sterilized by passing through a 0.2 μ m syringe filter. Cells were exposed to WR1065 at a final concentration of 40 μ M. At this concentration the drug is rapidly taken up by cells and becomes undetectable in the growth medium [28].

SOD2 siRNA transfection

BFS-1, BFS2C-SH02, and BFS2C-SH22 cells were grown to confluence in 150 mm dishes and were transfected with 100 nM, final concentration, *SOD2* or negative control (NC) short interfering RNA (siRNA, Ambion, Foster City, CA, USA). The RNA sequence was 5' AAG GAA CAA CAG GCC TTA TTC 3'. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen Life Technologies) according to manufacturer's instructions. Briefly, siRNA oligomer was diluted in 1.5 ml serum-free medium. Thirty microliters of

Lipofectamine 2000 reagent was diluted in 1.5 ml of serum free medium, mixed gently, and then incubated for 5 min at room temperature. The siRNA oligomer was then combined with the diluted Lipofectamine 2000 and gently mixed followed by incubation for 20 min at room temperature to facilitate complex formation. Growth medium was aspirated from the dishes and the cells were washed with PBS at 37 °C to remove traces of serum. The siRNA-Lipofectamine 2000 complexes were added to the culture dishes and gently mixed to allow for a uniform distribution. Cells were incubated with the transfection complexes for 24 h under the normal growth conditions of 37 °C and 95% air/5% CO₂. Following this treatment cells were washed with PBS at 37 °C and fresh complete growth medium was added

SOD enzyme activity assay

SOD2 activity was measured using the Superoxide Dismutase Assay Kit from Trevigen (Gaithersburg, MD, USA) following the manufacturer's instructions. Briefly, BFS-1, BFS2C-SH02 and BFS2C-SH22 cells grown to confluence in 150 mm dishes were harvested by scraping on ice and lysed with 350 µl of lysis solution. The resulting suspension was then centrifuged at 14,000g for 5 min at 4 °C and transferred to a clean 1.5 ml tube. Protein concentrations were determined following the standard Bradford assay and were adjusted to 5 µg/µl with lysis buffer. Activity was measured at room temperature using a colorimetric assay. Superoxide ions generated from the catalytic conversion of xanthine to uric acid and hydrogen peroxide by xanthine oxidase converts nitroblue tetrazolium (NBT) to NBT-diformazan. NBT-diformazan absorbs light at 550 nm. The extent of reduction in the appearance of NBT-diformazan is a measure of total SOD activity. Activity was measured using 50–500 µg of total cellular protein in the presence of 5 mM sodium cyanide (NaCN) (Sigma-Aldrich, St. Louis, MO, USA) to inhibit CuZnSOD (SOD1) activity. Absorbance changes were recorded for 5 min and the rate of increase in absorbance units per minute was calculated for each experimental sample and a negative control, which contained all of the reaction components except the cell lysate. The percentage inhibition was calculated and plotted as a function of protein concentration for each treatment condition. The highest maximum percentage inhibition for each group of sample curves to be compared was determined and used to calculate the amount of protein that inhibited NBT reduction by 50% of this maximum value. The SOD2 activities in U/mg protein were then calculated.

Micronucleus assay

The effects of WR1065 and low dose radiation exposure on chromosomal integrity in BFS-1 WT, BFS2C-SH02 and BFS2C-SH22 cells following a subsequent 2 Gy exposure were investigated by measuring the frequency of micronucleus formation using a cytokinesis block technique by Fenech described in detail elsewhere [29]. Briefly, 10⁶ sham- or drug/radiation-treated cells were plated in T-25 culture flasks in the presence of 1.5 µg/ml cytochalasin B and incubated for 48 h at 37 °C. At this concentration cytochalasin B is not toxic to any of the three cell lines. After incubation, the cells were harvested by trypsinization and then fixed with cold methanol:acetic acid (3:1), and stored at –20 °C. Slides were prepared and stained with acridine orange (0.01% in PBS) and examined under a fluorescent microscope (60×, dual band filter). At least 1000 binucleated cells with well-defined cytoplasm were scored for the presence of micronuclei. The frequency of micronucleus formation was calculated as the ratio of the number of binucleate cells containing micronuclei relative to the total number of binucleate cells scored.

Statistical analysis

Means and standard errors were calculated for all data points from at least three independent experiments. Pairwise comparisons of SOD2 activities and micronucleus frequencies

between each of the experimental conditions were performed using a Student's two-tailed *t* test.

Results

Characterization of BFS2C-SH22 cells

Following transfection of BFS2C-SH02 (TNFR1⁻) cells with a TRCN0000012322 TNFR2 targeting plasmid DNA, a number of stable transfectants were selected with puromycin and were designated BFS2C-SH18 through 22. Expression of TNFR2 in each of these clones was confirmed by quantitative PCR and the data are presented in Fig. 1 for comparison. The clone BFS2C-SH22 exhibited the greatest reduction in TNFR2 expression which was found to be about 50% of the level observed in BFS2C-SH02 cells. These cells, along with BFS-1 wild type and BFS2C-SH02 (TNFR1⁻) cells were used in all subsequent experiments to determine and contrast the role of TNF receptors on both the low dose radiation-induced and the thiol-induced adaptive responses.

Induction of SOD2 enzymatic activity

In a recent report we determined that SOD2 enzyme activity reached maximal levels at about 24 h following exposure of cells to a 10 cGy dose of ionizing radiation [21]. Both the kinetics and magnitude of low dose radiation-induced changes in SOD2 activity mimicked that observed in either rodent and human cells exposed to either low or high doses of thiol-containing drugs [17–21]. BFS-1 WT, BFS2C-SH02, and BFS2C-SH22 cell cultures were exposed to a 10 cGy dose of ionizing radiation and were assayed immediately and 24 h later for SOD2 enzymatic activity. As presented in Fig. 2A, a 10 cGy radiation dose induced a robust response in BFS-1 and BFS2C-SH02 cells as evidenced by significant elevations in SOD2 activities 24 h later. In contrast, SOD2 activity was unaffected in BFS2C-SH22 cells exposed to 10 cGy when assayed immediately or 24 h later, with SOD2 levels remaining essentially at background control levels. All three cell lines, however, exhibited significant elevations in SOD2 activities over background 24 h following their exposure for 30 min to WR1065 at a dose of 40 μ M (see Fig. 2B).

Low dose radiation-induced effects on SOD2 enzymatic activity and micronuclei formation as a function of cellular TNFR status

The inductive effect of a 10 cGy radiation exposure on the elevation of SOD2 activity in wild type BFS-1 and BFS2C-SH02 cells could be completely inhibited by transfection with *SOD2* siRNA (see Fig. 3A and B). Transfection with negative control siRNA did not result in a significant reduction of SOD2 activity. In contrast, no change in SOD2 activity was observed in BFS2C-SH22 cells 24 h following exposure to 10 cGy (see Fig. 3C). As shown in Fig. 3D, E and F, a 2 Gy dose of radiation was effective in significantly inducing elevations in micronuclei frequencies over both control and cells exposed to 10 cGy only. However, cells first exposed to 10 cGy and then challenged with 2 Gy 24 h later when SOD2 activity levels were maximal, exhibited a significant reduction in micronuclei formation. Cells transfected with NC siRNA were likewise still protected against radiation-induced micronuclei formation. Transfection of BFS-1 and BFS2C-SH02 cells with *SOD2* siRNA completely inhibited both the adaptive protective effect of the 10 cGy radiation dose against micronuclei formation and the elevation of endogenous SOD2 activity.

The TNFR1,2 knockout BFS2C-SH22 cells, in contrast, exhibited neither an elevation in SOD2 activity nor a significant response to *SOD2* siRNA transfection (see Fig. 3C). Consistent with the lack of an elevation in SOD2 activity in these cells 24 h following an exposure to 10 cGy, the BFS2C-SH22 cells also failed to exhibit any evidence of an

adaptive response regarding protection against micronuclei formation following a 2 Gy exposure (see Fig. 3F).

WR1065-induced effects on SOD2 enzymatic activity and micronuclei formation as a function of TNFR status

As observed following exposure to low dose ionizing radiation, SOD2 activity in BFS-1 cells was significantly elevated 24 h following WR1065 exposure, and this elevation was completely inhibited in cells transfected with *SOD2* siRNA (see Fig. 4A). When irradiated with a 2 Gy dose 24 h following WR1065 exposure, at which time SOD2 activity levels were significantly elevated, radiation-induced micronuclei formation was significantly inhibited to levels observed in unirradiated control cells (see Fig. 4D). As described earlier for the low dose radiation-induced adaptive response, NC siRNA transfection had no inhibitory effect on the thiol-induced adaptive response, while the effect was completely abolished in BFS-1 WT cells transfected with *SOD2* siRNA (see Fig. 4D).

Both BFS2C-SH02 (TNFR1⁻) and BFS2C-SH22 (TNFR1⁻R2⁻) cells were also capable of exhibiting a thiol-induced adaptive response characterized by both an elevation in SOD2 activity and resistance to radiation-induced micronuclei formation. As described in Fig. 4B and C, exposure to 40 μ M WR1065 resulted in a robust elevation in SOD2 activity 24 h later. Likewise, pretreatment with WR1065 also resulted in a significant protection against radiation-induced micronuclei formation when cells were irradiated with 2 Gy 24 h later when SOD2 activity was significantly elevated (see Fig. 4E and F). The thiol-induced adaptive response was also completely inhibited in BFS2C-SH22 cells transfected with *SOD2* siRNA.

Discussion

The radiation-induced adaptive response and the thiol-induced delayed radioprotective effect have both been investigated and found to share several similarities including the kinetics for the development and magnitude of each respective response, along with the strong correlation of changes in endogenous SOD2 enzymatic activities with these responses [4–6, 17–21]. While radiation-induced TNF α signaling has been implicated in the low dose radiation-adaptive response, its role in the thiol-induced radioprotective effect has not been investigated until now. To help address this issue we had to utilize a wild type cell line and cell lines defective in TNFR1 only and in both TNF receptors, 1 and 2 (Fig. 1), because it is well known that NF κ B activation can be facilitated through TNF signaling via either receptor [12–16]. The biological endpoint chosen for study was micronuclei formation, a marker for chromosome damage that is a reflection of genomic instability and a well-accepted marker for genotoxicity testing [32, 33].

TNF α is a multifunctional cytokine that expresses a variety of functions in many different cell types. It exerts its biological activity by binding to two receptors designated TNFR1 (p55TNFR) and TNFR2 (p75TNFR). These TNF receptors do not exhibit any metabolic capability, but must bind intracellular proteins to control subsequent signaling processes. While the complexity of the TNF signaling pathway includes both cell survival and death signals, TNFR1 and TNFR2 both can mediate prosurvival signals and can lead to the activation of NF κ B [31, 32]. A mechanism of action identified as underlying this effect is the activation of the TNF signaling pathway as a result of radiation-induced free radical formation which leads to stimulation of the NF κ B Inducing Kinase (NIK). NIK, in turn, affects phosphorylation of I κ B α leading to its degradation and the subsequent activation of NF κ B [33, 34]. NF κ B activation results in its translocation into the nucleus and its subsequent binding to an intronic NF κ B element in the *SOD2* gene which results in elevated gene expression [7]. Furthermore, it has been demonstrated that direct exposure to

exogenously administered TNF α , 20 to 24 h prior to a large challenge dose of ionizing radiation, has resulted in both enhanced SOD2 activity and associated cytoprotective effect in mammalian cells and animal systems comparable to that seen following a low dose radiation exposure [9, 17, 35]. It is with this context that we investigated the role of TNF receptors in both the low dose radiation- and thiol-induced adaptive responses.

As described in Fig. 2A, BFS-1 WT and BFS2C-SH02 TNFR1⁻ cells exposed to a 10 cGy dose of radiation exhibited a significant and robust elevation in SOD2 activity 24 h later. In contrast, BFS2C-SH22 TNFR1⁻R2⁻ cells did not exhibit any such increase in SOD2 activity consistent with the TNF α signaling model described above. Elevation of SOD2 activity following exposure to low dose irradiation is dependent upon a functional TNF α signaling pathway. WR1065, the free thiol form of amifostine, induces *SOD2* gene expression resulting in significant elevation in SOD2 activity 24 h later [22–24]. The mechanism of action underlying this phenomenon is the direct thiol-induced reduction of cysteine disulfide bonds in the p50 and p65 subunits of NF κ B [24, 25] that results in its activation and translocation into the nucleus [22–24]. This in turn results, as described for the TNF α signaling model, in the binding of NF κ B to an intronic NF κ B element in *SOD2* resulting in elevated gene expression and enzymatic activity [7]. Treatment of cells with the NF κ B inhibitors BAY 11-7082 [18] and Helenalin [19], or exposure of cells stably transfected with a mutant I κ B α gene to inhibit inducible phosphorylation of serine residues 32 and 36 thus preventing subsequent ligand-induced degradation required for NF κ B activation [18], completely inhibits induction of SOD2. All three BFS cell lines exposed to WR1065 exhibited significant elevation in SOD2 activity 24 h later (see Fig. 2B). While the effect was most robust in the BFS-1 cell line, elevated SOD2 levels in the BFS2C-SH02 and BFS2C-SH22 cell lines were also observed to reach significance demonstrating an independence of the TNF signaling pathway in the thiol-induced adaptive response.

The importance of SOD2 activity in both the low dose radiation- and thiol-induced adaptive responses utilizing micronuclei formation as an endpoint was further demonstrated by measuring the consequences of *SOD2* gene expression inhibition with *SOD2* siRNA. Fig. 3A and B demonstrate that transfection with *SOD2* siRNA completely inhibits the inductive effect of 10 cGy on elevation of SOD2 activity as measured 24 h later along with a concomitant abolition of the protective effect against radiation-induced micronuclei formation (See Fig. 3D and E). BFS2C-SH22 cells in which both TNF receptors were defective, failed to exhibit either a significant elevation in SOD2 enzyme activity following a 10 cGy exposure or an adaptive response as measured by a reduction in micronuclei frequency following irradiation with 2 Gy (see Fig. 3C and F). These data also demonstrate that inhibition of the TNFR1 receptor alone is not sufficient to inhibit the low dose radiation-induced adaptive response and further supports the importance of the TNF signaling pathway in the low dose radiation-induced adaptive response.

WR1065 exposure, in contrast to low dose irradiation, was effective in elevating SOD2 enzymatic activities in all three cell lines irrespective of their TNF receptor status supporting the hypothesis that direct activation of NF κ B activation by a thiol is sufficient to induce a SOD2 mediated adaptive response. Transfection with *SOD2* siRNA significantly inhibited not only this elevation in SOD2 activity (see Fig. 4A and C) but also in the concomitant protection against radiation-induced micronuclei formation (see Figs. 4D and F). These data demonstrate that the SOD2-mediated low dose radiation- and the thiol-induced adaptive responses, while differing in their dependence upon TNF signaling, share a commonality following the activation of NF κ B and subsequent effects on *SOD2* gene expression and enzymatic activities.

From the earliest reports regarding the identification of the low dose radiation-induced adaptive response, research in the field has been plagued by the variability of both the existence and the magnitude of the effect as a function of cell systems and endpoints evaluated, as well as the inconsistency observed between similar cell systems monitored in different individuals. To date, SOD2 has been one important element that has been identified as a mediator of a radiation-induced adaptive response. The linkage between free radical formation following low dose radiation exposure and the subsequent induction of the TNF α signaling process leading to NF κ B activation and elevation of *SOD2* gene expression offers a reasonable and highly testable mechanism underlying the development of enhanced radiation resistance characterized by the concept of low dose radiation-induced adaptive responses. It also suggests that subtle changes in TNF α signaling involving interactions with its two receptors (TNFR1 and 2) and downstream events culminating at the point of NF κ B activation could account for the variability observed in the expression of the adaptive response as a function of not only cell type, but also the source individuals from whom they are obtained. The independence of the thiol-induced SOD2 mediated adaptive response on the TNF α signaling pathway suggests that this phenomenon would be more universally observed presumably requiring only the presence of a functional NF κ B signaling pathway.

Within the paradigm of SOD2-mediated adaptive responses, there are several important implications. First, the effect appears to be unique to only SOD2 and not SOD1 or 3 [21]. Second, SOD2 is localized only within the mitochondria, while SOD1 is found in the cytoplasm of cells and SOD3 in the extracellular spaces [36]. This implies that the mitochondria represent the key subcellular organelle that is involved in the expression of the adaptive response. Third, all of the SODs appear to exhibit a single enzymatic activity, the dismutation of superoxide anion into hydrogen peroxide, which in turn through the action of catalase or glutathione peroxidase is converted to water and oxygen. This implies that superoxide anion formed in the mitochondrion as a result of a stress-inducing insult such as ionizing radiation exposure is the target molecule which is acted upon in the SOD2-mediated adaptive response that results in the diminution of overall radiation damage. Superoxide anion is not a strong oxidant, but it is known to be a potent precursor of other reactive oxygen species (ROS) and an effective initiator of ROS mediated cascade process(es) [37]. An example of such a process is a phenomenon known as ROS-induced ROS release (RIRR), in which mitochondria respond to elevated ROS concentrations by increasing their own ROS production [38–40]. Finally, if superoxide anion is the initiator molecule for the cascade process whose origin is within the mitochondrion, only SOD2 would be expected to exhibit a modulating effect on the process. Increased ROS within the mitochondria is known to lead to mitochondrial depolarization via activation of the mitochondrial permeability transition pore, which in turn can produce a short-lived burst of ROS originating from the mitochondrial transport chain [39]. ROS-induced opening of the inner mitochondrial membrane anion channel could also lead to an enhanced level of mitochondrial electron transport chain derived ROS. The implication of these processes is that the development of an ROS cascade will result in a large variety of different radical and oxidative molecules, of which superoxide anion will account for only a small subset. The ability of SOD1 or SOD3 to modulate such a cascade when it is released into the cytoplasm or extracellular spaces would be very limited due to the specificity of their substrate being limited only to superoxide anion. SOD2 in contrast, by virtue of its localization within the mitochondrion, would be expected to be very effective in inhibiting the initiation of any mitochondrial localized superoxide anion induced ROS cascade.

The SOD2-mediated adaptive response can be initiated by both low dose exposures of cells to ionizing radiation that induce oxidative damage, and by thiol-containing drugs such as WR1065 that are effective reducing agents. Both classes of agents appear to share the same pathway following activation of NF κ B that results in elevated SOD2 enzymatic activities

which reduce overall damage induced by a large challenge dose of ionizing radiation. Because of the uniqueness of SOD2's mode of action and localization, the implication of these phenomena is that these adaptive responses are the result of effectively inhibiting the induction of ROS damage cascades caused by superoxide anion formation within the mitochondria that can propagate and ultimately give rise to a component of the total radiation damage to the nuclear DNA of cells leading to enhanced genomic instability. It is proposed that the complexity of the TNF α signaling pathway component in the low dose radiation adaptive response might account for the variability in expression of this phenomenon.

Highlights

TNF receptor activity is associated with the low dose radiation adaptive response

Alterations in TNF signaling can account for variability in the adaptive response

The thiol-induced NF κ B-mediated adaptive response is independent of TNF signaling

The radiation-induced NF κ B-mediated adaptive response requires active TNF signaling

Elevated SOD2 mediates the low dose radiation- and thiol-induced adaptive responses

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TNFR2 Expression in TNFR1⁻ BFS2C

Fig. 1.

Effect of transfection of BFS2C-SH02 TNFR1⁻ cells with a TNFR2 targeting vector on TNFR2 expression resulting in five isolated clones designated: BFS2C-SH18, -SH19, -SH20, -SH21, -SH22. Stable transfectants were selected using puromycin and expression was confirmed by quantitative PCR. Error bars represent the standard error of the mean (SEM). BFS2C-SH22 (TNFR1⁻2⁻) cells were used in all subsequent experiments examining the effects of low dose radiation and thiol exposure on adaptive responses.

SOD2 Activity

Fig. 2.

A. Effect of low dose 10 cGy radiation exposure on manganese superoxide dismutase (SOD2) activity immediately and 24 h later in wild type (WT) BFS-1, TNFR1⁻ BFS2C-SH02, and TNFR1⁻R2⁻) BFS2C-SH22 murine fibrosarcoma cells. Each experiment was repeated three times and error bars represent the SEM. *P* values comparing cells exposed to 10 cGy to their respective unirradiated controls are presented. B. Effect of a 30 min exposure to 40 μ M concentration of WR1065 on SOD2 activity 24 h later in BFS-1, BFS2C-SH02 and BFS2C-SH22 cells. Each experiment was repeated three times and error bars represent the SEM. *P* values comparing cells exposed to WR1065 to their respective untreated controls are presented.

Radiation-Induced Adaptive Response

Fig. 3.

Effects of 10 cGy exposure and *SOD2* siRNA transfection on *SOD2* activity and micronucleus formation, respectively, in BFS-1 WT cells (A,D) BFS2C-SH02 (TNFR1^{-/-}) (B,E) and BFS2C-SH22 (TNFR1^{R2}) cells (C,F). *SOD2* activity was measured immediately and 24 h following irradiation in cells that were mock, negative control (NC), or *SOD2* siRNA transfected. Percentage of micronuclei was determined in untreated control cells, cells exposed to 10 cGy or 2 Gy, and mock, NC siRNA, and *SOD2* siRNA transfected cells exposed to 10 cGy followed 24 h later with 2 Gy. Experiments were repeated three times and error bars represent the SEM. *P* values were obtained for comparisons between single radiation dose conditions compared and their respective untreated controls. *P* values

were also obtained for mock, NC, and *SOD2* siRNA transfected cells exposed to 10 cGy followed 24 h later with 2 Gy as compared to non-transfected cells exposed to 2 Gy only.

Thiol-Induced Adaptive Response

Fig. 4.

Effects of 40 μM WR1065 exposure and SOD2 siRNA transfection on SOD2 activity and micronucleus formation, respectively, in BFS-1 WT (A,D), BFS2C-SH02 (TNFR1^{-/-}) (B,E) and BFS2C-SH22 (TNFR1^{-/-}R2^{-/-}) cells (C,F). SOD2 activity was measured 24 h following 40 μM WR1065 exposure in cells that were mock, negative control (NC), or SOD2 siRNA transfected. Percentage of micronuclei was determined in untreated control cells, cells exposed to 2 Gy, and mock, NC siRNA, and SOD2 siRNA transfected cells exposed to 40 μM WR1065 followed 24 h later with 2 Gy. Experiments were repeated three times and error bars represent the SEM. *P* values were obtained for comparisons between cells exposed to 2 Gy and their respective untreated control. *P* values were also obtained for

mock, NC siRNA, and SOD2 siRNA transfected cells treated with 40 μ M WR1065 and analyzed 24 h later as compared to non-transfected cells, and cells treated with WR1065 and irradiated with 2 Gy 24 h later as compared to non-transfected cells exposed to 2 Gy only.