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Long-Term Consequences of Radiation-Induced Bystander Effects Depend on Radiation Quality and Dose and Correlate with Oxidative Stress

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Abstract

Widespread evidence indicates that exposure of cell populations to ionizing radiation results in significant biological changes in both the irradiated and nonirradiated bystander cells in the population. We investigated the role of radiation quality, or linear energy transfer (LET), and radiation dose in the propagation of stressful effects in the progeny of bystander cells. Confluent normal human cell cultures were exposed to low or high doses of 1 GeV/u iron ions (LET ~ 151 keV/μm), 600 MeV/u silicon ions (LET ~ 51 keV/μm), or 1 GeV protons (LET ~ 0.2 keV/μm). Within minutes after irradiation, the cells were trypsinized and co-cultured with nonirradiated cells for 5 h. During this time, irradiated and nonirradiated cells were grown on either side of an insert with 3-μm pores. Nonirradiated cells were then harvested and allowed to grow for 20 generations. Relative to controls, the progeny of bystander cells that were co-cultured with cells irradiated with iron or silicon ions, but not protons, exhibited reduced cloning efficiency and harbored higher levels of chromosomal damage, protein oxidation and lipid peroxidation. This correlated with decreased activity of antioxidant enzymes, inactivation of the redox-sensitive metabolic enzyme aconitase, and altered translation of proteins encoded by mitochondrial DNA. Together, the results demonstrate that the long-term consequences of the induced nontargeted effects greatly depend on the quality and dose of the radiation and involve persistent oxidative stress due to induced perturbations in oxidative metabolism. They are relevant to estimates of health risks from exposures to space radiation and the emergence of second malignancies after radiotherapy.

INTRODUCTION

Increasing experimental evidence indicates that ionizing radiation induces important biological effects, including DNA damage, in cells that do not receive direct nuclear traversal (1). Bystander cells in the vicinity of directly irradiated cells or that are recipients of growth medium from irradiated cell cultures can also respond to the radiation exposure [reviewed in (2, 3)]. The persistence of stressful bystander effects for many generations after irradiation has been considered relevant for the assessment of human health risks associated with ionizing radiation exposure (4). Although delayed detrimental effects induced by radiation in bystander cells have been observed *in vitro* and *in vivo* [reviewed in (5, 6)], the role of radiation quality or linear energy transfer (LET) and dose in their induction remains

unclear. While conflicting results on the induction of bystander effects from low-LET radiation (e.g. X and γ rays) have been reported (7, 8), their expression after cellular exposure to α particles, a high-LET radiation, is well established (1). However, characterization of bystander effects in cell populations exposed to high-charge and high-energy (HZE) particles, another type of high-LET radiation, is only beginning to emerge (9–11). The occurrence of delayed stressful effects in the progeny of bystander cells from cultures exposed to HZE particles and/or protons would have significant relevance to space exploration. The lack of clear knowledge about these responses has been singled out by the U.S. National Academies of Science as a limiting factor for predicting radiation risks associated with human space exploration (4).

In addition to its importance in radiation protection, the characterization of nontargeted effects induced by proton or HZE-particle radiation is relevant to cancer radiotherapy. Due to the advantages in their physical dose distribution, protons and heavy ions (e.g. carbon ions) are used in radiotherapy to treat malignant tumors [reviewed in (12, 13)]. Studies on the communication of signaling events between irradiated and bystander cells are therefore extremely important. Specifically, transmission of stressful effects from irradiated tumor or normal cells to bystander normal cells and the persistence of such effects in their progeny would have profound implications for long-term health risks, including the emergence of second malignancies (14).

Using a Transwell® insert co-culture system, here we investigated the impact of LET and dose of radiation in the induction of long-term stressful effects, including genetic damage and perturbation of oxidative metabolism, in the progeny of bystander normal human cells that had been in co-culture with cells exposed to low or high mean doses from heavy ions or protons. Our experimental system allowed us to investigate HZE-particle-induced nontargeted effects in the absence of δ rays and secondary fragmentation products that may modulate signaling events elicited by the primary impacting particle. This novel strategy, together with ongoing studies from other laboratories, will increase our knowledge of the nontargeted biological effects triggered by different types of radiation and also the cross-talk between the induced signaling events.

MATERIALS AND METHODS

Cells

Normal human diploid skin fibroblasts (AG1522) were obtained from the Genetic Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). Cells at passage 10–12 were grown in Eagle's minimum essential medium (CellGro) supplemented with 12.5% heat-inactivated (56°C, 30 min) fetal calf serum (FCS), 200 mM L-alanyl-L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (Sigma). The cells were routinely maintained at 37°C in a humidified incubator with 5% CO₂ in air. For experiments, cells were seeded at a density that allowed them to reach the density-inhibited state within 5 days. They were then fed twice on alternate days, and experiments were initiated 24–48 h after the last feeding. Under these conditions, 90–98% of the cells were in G₀/G₁ phase of the cell cycle as determined by [³H]-thymidine uptake and/or flow cytometry (15, 16). Synchronization of the cells in G₀/G₁ phase by density inhibition eliminates complications in interpretation of the results that arise from changes in the cellular response to ionizing radiation at different phases of the cell cycle (17).

Irradiation

Iron-ion (1 GeV/u ⁵⁶Fe²⁶⁺), silicon-ion (600 MeV/u ²⁸Si¹⁴⁺) and proton (1 GeV ¹H⁺) irradiations were conducted at the NASA Space Radiation Laboratory (NSRL) located at the

Brookhaven National Laboratory (Upton, NY) (description of the facility and radiation beam information available at www.bnl.gov/medical/NASA/CAD/Bragg_Curves.asp) at dose rates ranging from 0.1 to 1 Gy/min, depending on the dose. The culture flasks were positioned perpendicular to the beam so that the irradiating particles first impacted the plastic of the culture vessel, followed by the adherent cells and then the growth medium. At the place where they were positioned, the LET was estimated to be ~151 keV/ μ m, 51 keV/ μ m and 0.2 keV/ μ m for 1 GeV/u ^{56}Fe ions, 600 MeV/u ^{28}Si ions and 1 GeV ^1H ions, respectively. The flasks were filled to capacity with growth medium that was pH- and temperature-equilibrated 3 h before the radiation exposure. This ensured that during the irradiation, temperature fluctuations were attenuated and the cells were immersed in medium, which alleviates changes in osmolarity and partial oxygen tension. The latter parameters greatly affect the cellular radiation response (18, 19). Control cells were sham-treated and handled in parallel with the test cultures. The experiments were carried out during different NSRL runs between 2007 and 2010; dosimetry was performed by the NSRL physics staff and is described further by Autsavapromporn *et al.* (20).

Cell Culture System

To examine radiation-induced nontargeted effects, a layered tissue culture system that allows isolation of pure bystander cells from contiguous irradiated cells was used. Briefly, AG1522 fibroblasts destined to be bystanders were seeded onto inverted Transwell® inserts with 3- μ m pores. After attachment, the inserts were inverted and placed into the wells of plates and cultured to confluence as described above. Irradiated cells derived from the confluent cultures maintained in flasks were harvested within 10 min after exposure to mean doses of 10 cGy–2 Gy from energetic iron ions, silicon ions or protons. The harvested cells were then seeded at confluent density on top of the insert with bystander cells growing at the bottom of it. Within 2 h after plating, irradiated cells adhered and formed functional junctional channels with bystander cells, as was assessed by the transfer of calcein dye (21). Irradiated and bystander cells may also communicate with each other through diffusible factors transferred across the pores of the membrane. The directly irradiated and bystander cells were left in co-culture for a total of 5 h. Subsequently, bystander cells were harvested, grown and serially subcultured for progeny studies upon reaching the confluent state. To include all the progeny cells derived from the initial bystander cell population, at each passage the cells were subcultured in increasingly larger flasks. After 20 population doublings that occurred in ~5 weeks, the cells were harvested and assayed for different end points. In this experimental strategy, bystander cells that are co-cultured with irradiated cells are not traversed by δ rays or secondary fragmentation products, nor would they be affected by activated growth medium.

Plating Efficiency

The plating efficiency (PE) was determined by the colony formation assay (22). Briefly, cells were trypsinized and seeded in 100-mm dishes at numbers that resulted in ~150 clonogenic cells per dish. Three replicates were done for each experimental point. After an incubation period of 12 days, the cells were rinsed with PBS, fixed in 95% ethanol and stained with crystal violet. Macroscopic colonies with more than 50 cells were counted. Each graph in the Results is representative of at least three separate experiments, and the results are reported as the mean $\pm$ SEM of the ratio of the PE of the progeny of bystander cells that were co-cultured with irradiated cells divided by the PE of the progeny of bystander cells that were co-cultured with sham-irradiated cells. The SEM were calculated by applying the rules of error propagation, and comparisons between treatment groups and controls were performed using Student's *t* test. A *P* value of ≤ 0.05 between groups was considered significant.

Micronucleus Formation

Radiation-induced DNA damage was assessed by measuring the frequency of micronucleus formation by the cytokinesis-block technique (23). Briefly, 2×10^4 cells were seeded in chamber flaskettes (Nalge Nunc International) in the presence of 2 $\mu\text{g/ml}$ cytochalasin B (Sigma). At this concentration, cytochalasin B was not toxic to the cells. After 72 h incubation, the cells were rinsed in PBS, fixed in ethanol, stained with Hoechst 33342 (1 $\mu\text{g/ml}$ in PBS), and viewed with a fluorescence microscope. At least 1000 binucleated cells per treatment in each experiment were examined. The fraction of micronucleated cells and the number of micronuclei per micronucleated cell was evaluated. For all treatments, the fraction of binucleated cells in the population was ~40%. Each graph is representative of at least three separate experiments, and Poisson statistics was used to calculate the standard errors associated with the percentage of micronucleated cells in the total number of binucleated cells scored. Evaluation of the frequency of micronucleus formation yielded a similar pattern of results as the fraction (%) of micronucleated cells reported in the Results. Comparisons between treatment groups and respective controls were performed using the Pearson's χ^2 test. A P value of ≤ 0.05 between groups was considered significant.

Western Blot Analyses

The cells were harvested by trypsinization, pelleted, rinsed in PBS, repelleted and lysed in chilled radio-immune precipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM NaF, 5mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] (Sigma) supplemented with protease and phosphatase inhibitor cocktails (Sigma). Protein concentrations were determined using the calorimetric Lowry assay (24) (Bio-Rad). Proteins were analyzed by SDS-PAGE followed by immunoblotting. Primary antibodies against catalase (Santa Cruz), copper-zinc superoxide dismutase (CuZnSOD) (Santa Cruz), manganese superoxide dismutase (MnSOD) (Upstate), glutathione peroxidase (GPx) (anti-GPx-1/2 from Santa Cruz), and human aconitase (unpublished), respectively, were used. Secondary antibodies conjugated with horseradish peroxidase and the enhanced chemiluminescence system from GE Healthcare was used for protein detection. Luminescence was determined by exposure to X-ray film, and densitometry analysis was performed with an Epson scanner and the National Institutes of Health Image J software (NIH Research Services Branch, Bethesda, MD). Staining with Ponceau S Red (Sigma) (25) or reaction of goat anti-rabbit immunoglobulin G (sc 2030, Santa Cruz) with a protein of ~30 kDa was used as loading control.

Oxidized Proteins

When proteins are oxidized by reactive oxygen species (ROS), some amino acids are modified, generating carbonyl groups. The latter can react with 2,4-dinitrophenyl hydrazine (DNPH), which in turn is recognized by anti-2,4 dinitrophenol (DNP) antibodies on immunoblots. For experiments, the OxyBlot Oxidized Protein Detection Kit (Millipore) was used. Carbonyl groups were derivatized with DNPH using 20- μg protein samples denatured with 6% SDS. Negative controls were reacted with a derivatization-control solution. After incubation at room temperature for 15 min, neutralization solution was added to each tube, and samples were analyzed by SDS-PAGE. Amido Black and/or Ponceau S Red staining were used as loading control and confirmed equal loading of sample amounts (25, 26).

Lipid Peroxidation

4-Hydroxy-trans-2-nonenal (4-HNE) generated through the β -cleavage of hydroperoxides from ω -6 polyunsaturated fatty acids reacts with proteins and was detected by SDS-PAGE followed by immunoblotting with anti-4-HNE antibody (Millipore). Amido Black and/or

Ponceau S Red staining were used as loading control and confirmed equal loading of sample amounts (25, 26).

Antioxidant Enzyme Activity

The activities of superoxide dismutases (i.e. MnSOD and CuZn-SOD), catalase and glutathione peroxidase (GPx) were measured by a native in-gel activity assay (27). Briefly, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM NaF, 5mM EDTA, 1% NP40, and supplemented with protease and phosphatase inhibitor cocktails (Sigma). Proteins were extracted as described above and electrophoresed in 8–10% non-denaturing polyacrylamide gel. Enzyme activities were measured by incubating the gels in assay buffers in the dark unless otherwise indicated. To measure SOD activity, the gel was soaked in Buffer A [20 mM potassium phosphate monobasic (KH_2PO_4), 30 mM potassium phosphate dibasic (K_2HPO_4)] for 5–10 min at room temperature with gentle shaking. After disposal of Buffer A, the gel was immersed for 1 h in freshly made Buffer B [12.5 mg of 4-nitroblue tetrazolium (4-NBT) per 200 ml Buffer A]. Finally, the gel was incubated for 15 min in freshly made Buffer C [14 mM KH_2PO_4 , 20 mM K_2HPO_4 , and 0.028 mM riboflavin]. When exposed to light, riboflavin generates superoxide anions that react with the chromogenic substrate 4-NBT and develops a purple color everywhere except where the anion is removed by SOD enzyme activity. Thus the gel was exposed to room lighting (~1250 lux) until optimal contrast and intensity were achieved (<5 min).

Catalase activity was revealed by washing the gel quickly in water and then soaking it in Solution A (0.005% H_2O_2 in water) for 10 min. The gel was then rinsed with water and incubated in Solution B [1% FeCl_3 , 1% $\text{K}_3(\text{Fe}(\text{CN})_6)$ in water] to develop the activity band (28).

To evaluate GPx activity, the gel was soaked in Solution A (Tris 25 mM, pH 8.0) at room temperature for 5 min. After disposal of Solution A, the gel was incubated for 10 min in Solution B (7 mM glutathione in Solution A). Solution B was discarded and the gel was soaked in freshly prepared Solution C (0.0015% H_2O_2 in water) for 10 min. It was then rinsed twice for 2 min with water and incubated in Solution D [1% FeCl_3 , 1% $\text{K}_3(\text{Fe}(\text{CN})_6)$ in water] to develop the activity band (28). Enzyme activities were quantified by densitometric analyses as described above.

Aconitase Activity

A native in-gel activity assay was adopted. Briefly, harvested cells were rinsed in PBS containing 2 mM sodium citrate, suspended in 100 μl lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1% TX-100, 10% v/v glycerol, 2 mM sodium citrate, 10 U catalase) (Sigma) and incubated on ice for 30 min. Samples were subsequently centrifuged at 15,000g for 10 min at 4°C. The supernatants were fractionated in non-denaturing polyacrylamide gel, and aconitase activity was measured by incubating the gel in assay buffer [100 mM Tris-HCl, pH 8.0, 1 mM NADP, 2.5 mM cis-aconitic acid, 5 mM MgCl_2 , 1.2 mM 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 0.3 mM phenazine methosulfate, 5 U/ml isocitrate dehydrogenase] (Sigma) in the dark with gentle shaking for 30 min at 37°C. The gel was washed with dH_2O to stop the reaction.

Mitochondrial Protein Translation

Mitochondrial protein translation was assessed as described (29) by incorporation of ^{35}S -methionine in cells wherein cytoplasmic protein synthesis was inhibited by cycloheximide. Briefly, confluent cell cultures were washed twice in PBS, incubated in cysteine- and methionine-free Dulbecco's Modified Eagle Medium (Cellgro) for 10 min at 37°C under an atmosphere of 5% CO_2 in air and subsequently incubated for 1 h with cycloheximide (10 $\mu\text{g}/$

ml). Cells were then incubated with ^{35}S -methionine [$10\text{ }\mu\text{Ci}/\mu\text{l}$ ($370\text{ kBq}/\mu\text{l}$)] for 2 h, washed with PBS and trypsinized. Extracted proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining and autoradiography.

RESULTS

Stressful Biological Effects Persist in the Progeny of Bystander Cells that Had Been in Co-culture with Cells Exposed to High- but not Low-LET Radiation

To investigate the dependence of radiation-induced stressful effects in the progeny of bystander cells on radiation quality and dose, we evaluated the clonogenic potential of the progeny of bystander AG1522 normal human fibroblasts that had been in co-culture with confluent AG1522 cells that were exposed to low or high mean doses of energetic iron ions or protons. After 20 population doublings, the progeny of isolated bystander cells that had been in co-culture with cell populations exposed to 10 cGy from 1 GeV/u ^{56}Fe ions (LET $\sim 151\text{ keV}/\mu\text{m}$) showed, relative to the respective control, a 50% reduction in their cloning efficiency ($P < 0.005$). The progeny of bystander cells that were in co-culture with cells exposed to higher doses (2 Gy) showed a 70% reduction ($P < 0.001$) (Fig. 1A). These reductions were associated with an increase in micronucleus formation, a reflection of chromosomal damage. Compared to controls, the fraction of micronucleated cells in the progeny of bystander cells that had been in co-culture with cells exposed to a mean dose of 10 cGy from ^{56}Fe ions was increased by 1.7-fold (2.0 ± 0.3 compared to 1.2 ± 0.2 ; $P < 0.005$). In the progeny of bystander cells that were co-cultured with cells irradiated with 2 Gy, a 3-fold increase was observed (3.5 ± 0.5 compared to 1.2 ± 0.2 ; $P < 0.001$) (Fig. 1B). Furthermore, an increasing number of micronuclei per cell were detected (Fig. 1C).

Parallel experiments were conducted in the progeny of bystander cells that had been in co-culture with cells irradiated with 25 cGy or 2 Gy from 1 GeV ^1H ions (LET $\sim 0.2\text{ keV}/\mu\text{m}$). In contrast to the increased stress in the progeny of bystander cells that were co-cultured with ^{56}Fe -ion-irradiated cells, the co-culture of bystander cells with cells exposed to either low or high doses of energetic protons did not produce a decrease in the cloning efficiency or an increase in micronucleus frequency in the progeny of the bystander cells (Fig. 1D–F). However, a small increase in the fraction of micronucleated cells with 3 micronuclei or more was observed (Fig. 1F). Collectively, these data show that the LET of the radiation plays a prominent role in the expression and persistence of stressful effects in the progeny of bystander cells. They show that the magnitude of these effects is dependent on the dose delivered to the exposed cell population.

The effect of high-LET radiation on the induction of persistent stressful effects in the progeny of bystander cells was further supported by co-culture studies in which the irradiated cells were exposed to 600 MeV/u ^{28}Si ions (LET $\sim 51\text{ keV}/\mu\text{m}$). Consistent with the data in Fig. 1B, at 20 population doublings after co-culture, the fraction of micronucleated cells in the progeny of bystander cells that were co-cultured with cells exposed to 50 cGy was increased by 6-fold relative to control ($P \ll 0.001$; data not shown).

A Pro-oxidant-Based Mechanism is Associated with the Persistence of Stressful Effects in the Progeny of Bystander Cells that Had Been in Co-culture with Cells Exposed to High-LET Radiation

Though a burst of excess ROS is initially produced at the time of irradiation and is believed to persist for only microseconds or less, radiation-induced cellular oxidative stress may be prolonged due to long-term effects on oxidative metabolism (30). Exposure to ionizing radiation may affect respiratory complexes as well as mitochondrial and membrane oxidases (31), leading to excess ROS production, and may also disrupt antioxidant activity. ROS are

very reactive and their interaction with DNA, RNA, proteins and lipids can cause significant cellular damage. To investigate whether the excess DNA damage in the progeny of bystander cells (Fig. 1B) correlates with oxidative stress, we examined the level of protein carbonylation in the progeny of bystander cells that were co-cultured with cell populations exposed to either low or high doses from 1 GeV/u ^{56}Fe ions or 1 GeV protons. Protein carbonylation induced by ROS is a robust marker of oxidative stress and has been implicated in a multitude of physiological processes including aging and immune functions (32). Whereas protein carbonylation was strongly increased in the progeny of bystander cells that were co-cultured with iron-ion-irradiated cells (Fig. 2A), it was not appreciably affected in the progeny of bystander cells that were co-cultured with proton-irradiated cells (Fig. 2C).

To investigate the contribution of lipoxidation-derived reactive carbonyls to protein carbonylation (Fig. 2A), we examined the level of 4-HNE adducts in proteins from the progeny of bystander cells. Relative to the respective control, a dose-dependent increase in 4-HNE signal in the progeny of bystander cells that were co-cultured with iron-ion-irradiated cells was generally observed (Fig. 2B). No significant changes were detected in the progeny of bystander cells that were co-cultured with proton-irradiated cells (Fig. 2D).

Carbonylation is irreversible and unrepairable (33), and the observed increases (Fig. 2A and B) may disrupt protein secondary and tertiary structures with consequent loss of catalytic and structural protein functions.

An imbalance between ROS levels and antioxidant defense appears to be common to the expression of damage in bystander cells and long-term stressful effects in irradiated cells (34–36). Thus, to further understand the events underlying the above effects (Figs. 1, 2), we examined the modulation of the activity of the antioxidant enzymes MnSOD, CuZnSOD, catalase and glutathione peroxidase (GPx), in the progeny of bystander cells that had been in co-culture with cells exposed to low or high mean doses from iron ions or protons.

The SODs are metal-containing proteins that catalyze the removal of superoxides, generating hydrogen peroxide as a final product of the dismutation (37). Among the different mammalian isoforms of SOD, we focused on SOD1 (i.e. CuZnSOD), which is found in the cytoplasm and nucleus, and SOD2 (i.e. MnSOD), which is localized to the mitochondrial matrix (38). By contrast, catalases, which are largely found in peroxisomes and cytoplasm (37), are heme-containing enzymes that convert hydrogen peroxide to water and oxygen. Glutathione peroxidases are present in the cytoplasm and in the mitochondrial matrix (37); they remove peroxides by coupling their reduction with the oxidation of glutathione.

The representative data from three repeat experiments in Fig. 3 indicate that the progeny of bystander cells that were co-cultured with cells exposed to a mean dose of 10 cGy or 2 Gy from iron ions showed an overall reduction in the activity of MnSOD, CuZnSOD, catalase and GPx. Whereas MnSOD activity decreased by 25% ($P < 0.005$) and 40% ($P < 0.0005$) in the progeny of bystander cells that were co-cultured with cells exposed to a mean dose of 10 cGy or 2 Gy, respectively, CuZnSOD activity was reduced by 25% ($P < 0.001$) and 45% ($P < 0.005$) in these cells. The activities of catalase and GPx were reduced by 30% in the progeny of bystander cells that were co-cultured with cells exposed to a mean dose of 2 Gy ($P < 0.005$) (Fig. 3A). For MnSOD, CuZnSOD and catalase, but not GPx, the decrease in activity may be due to the observed decrease in protein level (Fig. 3B).

In agreement with the lack of detectable long-term effects on clonogenic potential, residual DNA damage, protein carbonylation, and lipid peroxidation in the progeny of bystander cells that had been in co-culture with cells exposed to either low (25 cGy) or high doses (2 Gy) of energetic protons, there were no significant changes in the activity or level of

MnSOD, CuZnSOD and GPx (Fig. 3C and D) in these cells. In the case of catalase, an increase (1.5-fold) in the protein level was observed. However, this did not correlate with an increase in enzyme activity (Fig. 3C). Therefore, these results further support the notion that the persistence of stressful effects in the progeny of bystander cells depends on radiation quality and dose. Compared to low-LET proton radiation, high-LET iron-ion radiation promoted in the progeny of bystander cells a sustained state of oxidative stress (Fig. 2A and B) that was associated with decreased antioxidant activity (Fig. 3A and B).

Mitochondrial Functions are Perturbed in the Progeny of Bystander Cells that Had Been in Co-culture with Cells Exposed to High- but not Low-LET Radiation

It is estimated that as much as 1% of the total oxygen consumed in a cell results in the formation of superoxide anions ($O_2^{\cdot-}$) generated mainly in mitochondria. Exposure to ionizing radiation increases superoxide production in an LET-dependent manner (39). An important mechanism of $O_2^{\cdot-}$ toxicity is its oxidation and resultant inactivation of iron-sulfur (Fe-S) proteins, such as the mitochondrial aconitase. Damage to the $[4Fe-4S]^{2+}$ cluster of aconitase leads to enzyme inactivation with concomitant release of iron, which in turn may promote generation of the highly reactive hydroxyl radicals ($\cdot OH$) through the Fenton reaction. Mitochondrial aconitase (ACO2) plays a key role in the tricarboxylic acid (TCA) cycle by catalyzing the reversible dehydration of citrate to isocitrate. The inhibition of ACO2 thereby affects energy production and overall cellular viability (40). The role of LET and dose of radiation in the modulation of the activity of ACO2 in the progeny of bystander cells has not been investigated and may serve as an indicator of their redox state.

Compared to controls, ACO2 activity was reduced by 65% ($P < 0.0001$) in the progeny of bystander cells that had been in co-culture with cells exposed to 2 Gy from iron ions (Fig. 4A). However, ACO2 protein levels remained unchanged (Fig. 4B), suggesting oxidative damage to the $[4Fe-4S]^{2+}$ cluster in the active site of the enzyme. Parallel experiments were conducted in the progeny of bystander cells that had been in co-culture with cells exposed to low (25 cGy) or high mean doses (2 Gy) from protons. Whereas the activity of ACO2 was increased in the progeny of bystander cells that were co-cultured with low-dose-irradiated cells, it was unchanged in the case of high doses (Fig. 4C). ACO2 protein levels were similar to those in the progeny of control cells (Fig. 4D).

Although most of the proteins in mitochondria are encoded by nuclear DNA, a few are encoded by mitochondrial DNA and are synthesized by a mitochondrial protein translation system (41). Mitochondrial DNA encodes for 13 proteins that are subunits of the protein complexes in the electron transport chain. To gain greater understanding of the mechanisms underlying the effect of radiation quality and dose on differential induction of oxidative stress in the progeny of bystander cells, we examined changes in the level of mitochondria-encoded proteins in cells wherein cytoplasmic protein synthesis was inhibited.

Relative to controls, at 20 population doublings after co-culture with cell populations irradiated with 2 Gy from energetic iron ions, an overall increase in mitochondria-translated proteins was observed in the progeny of the bystander cells (Fig. 5A). In contrast, similar levels of translation products were detected in the progeny of bystander cells that had been in co-culture with cells exposed to 0 or 2 Gy of 1 GeV protons (Fig. 5B). These data show that bystander cells from cultures exposed to HZE particles, but not protons, experience changes that may lead to altered mitochondrial functions in their progeny. Changes in the expression of mitochondria-encoded proteins likely affect respiratory chain complexes with ensuing consequences to normal cellular physiology (32).

DISCUSSION

During missions in deep space, only a small fraction of the cells in an astronaut's body would be traversed at any one time by an energetic particle. However, the expression of bystander effects suggests that a greater fraction of cells than those that have been directly irradiated may be at risk (42). Clearly, the persistence of stressful effects in bystander cells would affect human health risk estimates associated with space travel.

Although it has been shown *in vitro* and *in vivo* that radiation-induced stressful effects can be transmitted to the progeny of irradiated cells [reviewed in (5, 6)], few reports have examined the susceptibility of normal human cells to long-term effects induced by radiation in a bystander scenario. Among other factors, radiation quality (i.e. LET) and radiation dose likely influence the induction and the extent of these effects. The deposition of energy by high-LET radiation in a cell results in a large local dose that triggers gene expression patterns that are different from those observed after exposure to a similar dose of low-LET radiation (43, 44). Furthermore, studies suggest that the mechanisms underlying the short-term effects of low and high doses of radiation may be different (45). The effects of LET and dose of radiation on the induction of stressful effects in bystander cells and their persistence in their progeny remain unclear (9–11).

Here we show that in contrast to low-LET (0.2 keV/μm) proton radiation, high-LET iron- or silicon-ion radiation (~ 151 and ~ 51 keV/μm, respectively) was effective at inducing stressful effects in the progeny of bystander cells that were detectable at 20 population doublings after co-culture. Compared to control, the progeny of bystander cells that were co-cultured with cells exposed to low or high doses of 1 GeV/u iron ions or 600 MeV/u silicon ions showed a decrease in cloning efficiency and an increase in micronucleus formation (Fig. 1A–C). In contrast, the progeny of bystander cells that had been in co-culture with cells exposed to low or high doses of 1 GeV protons retained their ability to form colonies and expressed similar levels of micronuclei as the progeny of cells that had been in co-culture with sham-irradiated cells (Fig. 1D–F). The delayed reproductive death that manifested as reduced plating efficiency and the persistence of residual DNA damage in the progeny of bystander cells that were co-cultured with cells exposed to high-LET radiation may be due to *de novo* lethal mutations and/or chromosomal aberrations, characteristics of genomic instability (46). Together, our data demonstrate that the transmission of stressful effects in the progeny of bystander cells is LET-dependent. Moreover, the high-LET radiation-induced effects are dose-dependent (Fig. 1A–C).

The molecular mechanisms underlying the induction and persistence of detrimental effects in the progeny of bystander cells are not fully elucidated. The data from this study support the role of oxidative metabolism in the persistence of the induced stressful effects (Figs. 3 and 4). Redox-modulated events that likely result from a perturbation in oxidative metabolism (47) may be common to both the induction of bystander effects and the persistence of radiation-induced damage in the progeny of irradiated cells. Although the effect of LET on the yield of reactive radiolytic products has been characterized (48) and the prevalence of specific radiolysis species at the time of irradiation may potentially induce dissimilar effects in bystander cells (49, 50), the impact that the LET and dose of radiation may have on the induction of a sustained oxidative stress, which may lead to neoplastic transformation and degenerative conditions in the progeny of bystander cells, remains elusive.

Our study indicates that high- but not low-LET radiation induces enduring perturbations in oxidative metabolism of bystander cells. Twenty population doublings after co-culture with cells exposed to low (10 cGy) or high (2 Gy) mean doses of energetic iron ions, bystander

cells exhibited higher levels of protein oxidation (Fig. 2A) and lipid peroxidation (Fig. 2B). Decreased aconitase (Fig. 4A) and antioxidant enzyme activities (Fig. 3A) were also observed in these cells. The extent of the changes depended on the dose delivered to the irradiated cell population. Whereas at a mean dose of 2 Gy from 1 GeV/u iron ions, all the nuclei in an AG1522 monolayer cell culture are hit on average by ~11 particles (~15 cGy/hit), at a mean dose of 10 cGy, only ~50% of nuclei are hit by 1 particle (51). Significantly, the latter stressful effects did not occur in the progeny of bystander cells that had been in co-culture with cells exposed to low (25 cGy) or high (2 Gy) mean doses from energetic protons (Figs. 2C, 2D, 3C and 4C). The average hits per cell nucleus from mean doses of 25 cGy or 2 Gy from 1 GeV protons are ~1090 and 8700, respectively (51); however, the sparsely ionizing events induced by these hits did not trigger sustained effects in progeny cells similar to those of the densely ionizing iron-ion hits.

Carbonylation is an irreversible oxidative process that leads to unrepairable protein modifications (33), which can disrupt signaling events. It may also change the transport properties of lipid bilayers and the transmembrane potential of both plasma and nuclear membranes and cause the accumulation of cytotoxic products (52). Carbonylation of histones, the essential components of eukaryotic chromatin, has potentially severe consequences for the maintenance of genomic integrity (53). It alters chromatin compactness with significant consequences to DNA repair. Similarly, 4-HNE, a relatively stable end product of lipid peroxidation and a potent alkylating agent, can react with DNA and proteins, generating harmful adducts that affect cellular proliferation, differentiation and apoptosis [reviewed in (54)]. In sum, carbonylation events may impair the activity of key proteins essential for healthy survival (33) and correlate with the decreased cloning efficiency in the progeny of bystander cells observed in our studies (Fig. 1A).

The increase in protein oxidation and lipid peroxidation in distant progeny of bystander cells that were co-cultured with cells irradiated with iron ions is likely due to an increase in the level of ROS, which may arise from an effect on respiratory complexes, ROS-generating oxidases, and/or a disruption of antioxidant defenses (31). Our study showed a decrease of both mitochondrial and cytoplasmic antioxidant enzyme activities in these cells (Figs. 3A and 4A). It has been reported that cellular incubation with SOD or catalase significantly reduces the yield of micronuclei in bystander cells (35). Our data extend these observations and indicate that the persistent induction of micronuclei in the progeny of bystander cells (Fig. 1B and C) is associated with a reduction in antioxidant activity (Fig. 3A). Among the antioxidant enzymes investigated in this study, GPx was shown to play a major role in cellular detoxification of 4-HNE adducts (55); thus the reduction in GPx activity in the progeny of these bystander cells (Fig. 3A) may in part explain their increased level of lipid peroxidation (Fig. 2B).

Mitochondrial aconitase, a key enzyme of the TCA cycle, contains a $[4\text{Fe-4S}]^{2+}$ cluster in its active site that is highly susceptible to oxidation by ROS with consequent impairment in cellular energy metabolism (40). Given the reversible nature of the response of aconitase to alterations in redox status, a reduction in aconitase enzyme activity in progeny cells may be indicative of a regulated response to elevated oxidative stress (56). Inhibition of aconitase may serve to reduce the supply of NADH for electron transport, thereby limiting the production of free radical species (56). Nevertheless, the reduction of aconitase activity in the progeny of bystander cells that had been in co-culture with high LET-irradiated cells (Fig. 5A) was associated with an increase in protein oxidation (Fig. 2A) and lipid peroxidation (Fig. 2B), and a reduction in antioxidant enzyme activity (Fig. 3A) contributing to a persistent state of oxidative stress.

Human mitochondrial DNA encodes for 13 polypeptides that constitute the central core of the oxidative phosphorylation complexes located in the mitochondrial inner membrane (41). The correct expression of this small set of proteins is essential for energy production and for all those processes in which mitochondria play a role. In recent years it has become clear that defects of mitochondrial translation and protein assembly cause distinct clinical phenotypes (57). Compared to control, an overall upregulation of mitochondrial protein translation products was observed in the progeny of bystander cells that were co-cultured with cells exposed to high doses of energetic iron ions but not protons (Fig. 5). The increase in translated products may be due to an increase in mitochondrial content or ATP demand. However, compared to controls, no changes in mitochondria size/mass or the steady-state levels of ATP were observed in our studies (data not shown).

CONCLUSION

Our data demonstrate that the propagation of stressful effects in the progeny of bystander normal human cells is LET-dependent. The progeny of bystander cells that were co-cultured with cells exposed to low fluences from iron ions exhibited a condition of persistent oxidative stress that might have induced (or may have been the effect of) impaired mitochondrial functions. Such effects were not observed in the progeny of bystander cells that were co-cultured with cells exposed to protons. Moreover, the transmission of stressful effects induced by high-LET radiation in the progeny of bystander cells was dependent on the dose delivered to the irradiated cell populations.

Available databases on the long-term effects on the health of astronauts are likely to remain extremely limited in the near future. A better understanding of the biological responses to space radiation exposures in model experimental systems will help reduce the uncertainty in the assessment of the health risks to astronauts from low-level exposure to radiation during space travel. Our results provide a mechanistic insight into the long-term consequences to human health from exposure to energetic protons or HZE particles. The persistence of oxidative stress in the progeny of bystander cells may amplify the risk of carcinogenesis and degenerative diseases (58), including cognitive defects and cardiovascular disorders (59, 60). Furthermore, our studies are relevant to radiotherapy with protons or heavy ions and its long-term consequences (61).

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FIG. 1.

Differential effects of LET and dose of radiation on the persistence of stressful effects in the progeny of bystander AG1522 cells. (Panel A) Ratio of the plating efficiency (PE) of the progeny of bystander cells to the PE of respective control cells, (panel B) micronucleus formation, and (panel C) micronucleus distribution in the progeny of bystander cells that had been in co-culture 20 population doublings earlier with cell populations exposed to mean doses of 0 or 10 cGy or 2 Gy (200 cGy) from 1 GeV/u ^{56}Fe ions. (Panel D) Ratio of the plating efficiency (PE) of the progeny of bystander cells over the PE of respective control cells, (panel E) micronucleus formation, and (panel F) micronucleus distribution in the progeny of bystander cells that had been in co-culture with cell populations exposed to mean doses of 0 or 25 cGy or 2 Gy (200 cGy) from 1 GeV protons. The results indicate that co-culture of bystander cells with high- but not low-LET-irradiated cells induced persistent stressful effects in the progeny of the bystander cells that depended on the dose delivered to the irradiated cell population. For all treatments, the percentage of binucleated cells in the examined populations was ~40% after incubation with cytochalasin B. Each graph is representative of at least three separate experiments.

FIG. 2.

Persistence of oxidative stress in the progeny of bystander cells 20 population doublings after co-culture with irradiated cells is dependent on the quality and dose of the radiation. (Panel A) Protein carbonylation and (panel B) lipid peroxidation (i.e. 4-HNE protein adduct accumulation) as revealed by immunoblotting in confluent bystander cells that had been in co-culture with cell populations exposed to mean doses of 0 or 10 cGy or 2 Gy (200 cGy) from 1 GeV/u ^{56}Fe ions. Relative to controls, the progeny of bystander cells exhibited higher levels of protein carbonylation and lipid peroxidation. (Panel C) Protein oxidation and (panel D) lipid peroxidation in the progeny of bystander cells that had been in co-culture with cell populations exposed to mean doses of 0 or 25 cGy or 2 Gy (200 cGy) from 1 GeV protons. Twenty population doublings after co-culture, bystander cells exhibited levels of protein carbonylation and 4-HNE protein adduct accumulation similar to those of control cells. Each immunoblot is representative of three separate experiments.

FIG. 3.

The modulation of antioxidant enzymes in the progeny of bystander cells 20 population doublings after co-culture with irradiated cells depends on radiation quality. Analyses of antioxidant enzyme activities detected by in-gel assays (panel A) and of protein levels examined by Western blot analyses (panel B) in the progeny of bystander cells that had been in co-culture with cell populations exposed to mean doses of 0 or 10 cGy or 2 Gy (200 cGy) from 1 GeV/u ^{56}Fe ions. Panels C and D show antioxidant enzyme activities and protein levels in the progeny of bystander cells that had been in co-culture with cell populations exposed to mean doses of 0 or 25 cGy or 2 Gy (200 cGy) from 1 GeV protons. Staining with Ponceau S Red was used as loading control. Each immunoblot is representative of three separate experiments. In panels A and C, Fold change = relative change.

FIG. 4.

Modulation of the activity and protein level of aconitase in the progeny of bystander cells 20 population doublings after co-culture with cell populations exposed to radiations of different LET. (Panel A) Mitochondrial aconitase (ACO2) enzyme activity measured by in-gel assay and (panel B) ACO2 protein level determined by Western blot analyses in confluent bystander cells that had been in co-culture with cells exposed to mean doses of 0 or 10 cGy or 2 Gy (200 cGy) from 1 GeV/u ^{56}Fe ions. (Panel C) ACO2 activity and (panel D) protein level in bystander cells that were co-cultured with cell populations exposed to mean doses of 0 or 10 cGy or 2 Gy (200 cGy) from 1 GeV protons. Staining with Ponceau S Red was used as loading control. Each gel or immunoblot is representative of three separate experiments. In panels A and C, Fold change = relative change.

FIG. 5. Mitochondrial protein translation in the progeny of bystander cells 20 population doublings after co-culture with cell populations exposed to radiations with different LET. Protein translation products in the progeny of bystander cells that had been in co-culture with cells exposed to 0 or 2 Gy (200 cGy) from (panel A) 1 GeV/u ^{56}Fe ions or (panel B) 1 GeV protons. An overall increase in mitochondria-translated proteins (41) was observed in the progeny of bystander cells that had been in co-culture with iron ion- but not with proton-irradiated cells. Staining with Coomassie Blue was used as loading control. Each gel is representative of three separate experiments.