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Normal Human Fibroblasts Exposed to High- or Low-Dose Ionizing Radiation: Differential Effects on Mitochondrial Protein Import and Membrane Potential

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ABSTRACT

How oxidative metabolism modulates effects of ionizing radiation is incompletely understood. Because mitochondria participate in oxidative metabolism, we investigated the modulation of mitochondrial protein import and membrane potential ($\Delta\Psi$) in irradiated cells. Our data show that effects at low dose cannot be predicted from effects at high dose. When density-inhibited normal human fibroblasts were exposed to a toxic dose of 4 Gy, protein import into mitochondria isolated from these cells was decreased. In contrast, protein import into mitochondria isolated from low-dose–irradiated (10 cGy) cells was enhanced, suggesting that mitochondria may play a crucial role in low-dose–induced adaptive responses. At high dose, import defects were not solely due to changes in mitochondrial $\Delta\Psi$, and modulation of import was not tightly linked to the cellular capacity to repair radiation damage. Another striking observation is that in proliferating nonirradiated cells, mitochondrial protein import and $\Delta\Psi$ were regulated in a cell cycle–dependent manner, being lower in *S* phase than in G_1. Interestingly, when quiescent G_0/G_1 phase cells exposed to high-dose radiation were stimulated to proliferate, events associated with *S* phase, but not G_1, significantly affected import. The strategy described here may serve as novel end points to study radiation-induced effects. *Antioxid. Redox Signal.* 8, 1253–1261.

INTRODUCTION

IONIZING RADIATION–INDUCED CELLULAR DAMAGE results from direct and indirect effects. The direct effect is due to ionizations or excitations caused by the transfer of radiation energy from incident photons or particles to target molecules in their path. The indirect effect originates from free radicals and reactive oxygen species (ROS) produced primarily from the radiolysis of water near target molecules. Intracellular diffusion of the reactive species may then lead to covalent modifications of DNA, lipids, and proteins (9). These initiating physicochemical reactions occur within milliseconds after exposure and can result in biomolecular lesions within minutes (44). Regardless of the mechanism (direct or indirect), damage to DNA is considered to be the most important lesion produced during or shortly after exposure (12, 42, 52). In contrast, oxidative stress generated by radiation-induced perturbations in oxidative metabolism induces damage to critical biomolecules, including DNA, long after radiation exposure (46). Such a delayed effect may play a prominent role in radiation-induced genomic instability (23, 27), carcinogenesis, and the development of degenerative diseases, including atherosclerosis and premature aging (15, 33, 36). Studies of radiation effects on various organelle functions that contribute to oxidative metabolism are therefore critical for better understanding of

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mechanisms that can lead to prevention of radiation-induced degenerative effects.

Mitochondria mediate respiration, adenosine triphosphate (ATP) synthesis, and thermoregulation in eukaryotic cells. They also participate in numerous indispensable metabolic pathways (*e.g.*, synthesis of heme, nucleotides, lipids, and amino acids), mediate intracellular homeostasis of inorganic ions, and play a major role in apoptosis (24, 26, 45). They consume ~90% of the body's oxygen (14). Although they are the richest source of ROS, they also play an essential antioxidant function (45). Mitochondrial dysfunction, particularly in cells exposed to ionizing radiation, may therefore perturb oxidation–reduction reactions that determine the cellular redox environment and, by corollary, many cellular functions such as ion transport, gene expression, protein stability, and DNA repair (20).

Whereas mitochondrial signaling to radiation-induced apoptotic death is well characterized (17), the role of mitochondria in other radiation-induced effects/phenomena is only emerging. Our ongoing studies and those of others suggest that oxidative metabolism mediates adaptive and bystander responses induced by low-dose and low-fluence ionizing radiation, respectively (3, 8, 18). The expression of such responses challenges current radiation-protection recommendations, which are based on the assumption that the deleterious effects of ionizing radiation have no dose threshold and show a linear dose response (29, 51). Whereas the adaptive response is considered to attenuate radiation damage to cells, bystander responses are generally thought to amplify deleterious effects. These two processes could affect long-term effects, including carcinogenesis, in ways that deviate from the assumed linearity of the dose–response relation (8, 13, 40, 41). Understanding the mechanisms underlying these phenomena is currently of immense interest to the public, the scientific community, and regulatory agencies.

Protein import into mitochondria is a fundamental mechanism of mitochondrial biogenesis—maintenance and regeneration of mitochondria. Although mitochondria contain their own DNA and complete systems for its replication, transcription, and translation, they synthesize only a few (13 in humans) proteins. All other mitochondrial proteins are nuclear encoded and are synthesized on cytoplasmic ribosomes. These proteins must be transported from cytosol to the correct mitochondrial subcompartment (21). Translocation of proteins across the mitochondrial inner membrane into the matrix requires potential difference across this membrane. A disruption in the maintenance of membrane potential ($\Delta\Psi$) and/or protein import may significantly affect oxidative metabolism as well as short- and long-term effects of stressful agents such as ionizing radiation. In an effort to elucidate molecular and biochemical events underlying the role of oxidative metabolism in high- and low-dose radiation effects, we investigated the modulation of mitochondrial functions in irradiated normal human diploid fibroblasts. Specifically, we examined radiation-induced effects on mitochondrial protein import and membrane potential. In the context of these two processes, our data argue against the currently assumed linear dose–response relation.

MATERIALS AND METHODS

Cell culture

AG1522 normal human diploid skin fibroblasts were obtained from the Genetic Cell Repository at the Coriell Institute for Medical Research (Camden, NJ). Cells at passage 10–14 cultured in Eagle's Minimal Essential Medium supplemented with 12.5% heat-inactivated fetal calf serum, glutamine (2 m*M*), 100 units/ml penicillin, and 100 μg/ml streptomycin (growth medium) were used. They were maintained in a 37°C humidified incubator in an atmosphere of 5% CO_2 in air. Unless specified, cells were cultured to reach the density-inhibited state within 5 days after seeding. On reaching confluence, they were fed twice on alternate days with growth medium and were exposed to γ-rays 24–48 h after the last feeding. Under these conditions, 90–98% of the cells were in G_0/G_1 as determined by uptake of [^{3}H]-thymidine and/or flow-cytometric analyses. Synchronized cells were used to eliminate complications in interpretation of the results, which arise from changes in the cellular response to ionizing radiation at different phases of the cell cycle (47).

Irradiation conditions

For high-dose exposures, cell cultures were irradiated with γ-rays from a ^{137}Cs source at a dose rate of 3.3 Gy/min (J.L. Shepherd, Mark I, San Fernando, CA). For low doses, cells were exposed at 37°C in a custom-designed ^{137}Cs-irradiator (J.L. Shepherd, Model 28–8) at a dose rate of 0.2 cGy/h. Control cultures were sham-treated and handled in parallel with the test cultures.

Isolation of mitochondria

Mitochondria were isolated from cell cultures harvested at different times after irradiation or sham-manipulation, as described (35), with some modifications. In brief, ~40–50 × 10^6 cells per experimental group were harvested by trypsinization, followed by suspension in growth medium and rinsing with ice-cold phosphate-buffered saline (PBS). Pelleted cells were resuspended in 7.5 ml ice-cold hypotonic buffer (10 m*M* NaCl, 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl, pH 7.5) and incubated for 10 min at 4°C followed by Dounce homogenization (40 strokes). Mitochondria isolation buffer (525 m*M* mannitol, 175 m*M* sucrose, 12.5 m*M* ethylenetetraacetic acid (EDTA), 12.5 m*M* Tris-HCl, pH 7.5; 10.5 ml) was then added to the homogenate and centrifuged at 800 *g* for 5 min. The pellet was subjected to a repeat of these isolation steps. The hypotonic and mitochondria isolation buffers were supplemented, immediately before use, with 1 m*M* PMSF (phenylmethylsulfonyl fluoride; Sigma Chemical Co., St. Louis, MO) and 1 μg/ml protease inhibitor cocktail prepared in DMSO (5 m*M* E-64; 12.5 m*M* leupeptin; 12.5 m*M* pepstatin; 5 m*M* bestatin; 250 m*M* 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride) (MP Biomedicals, Irvine, CA). Pooled supernatants from each of the repeated isolation steps were centrifuged at 10,000 *g* for 10 min. Mitochondrial pellets were resuspended in 20 m*M* Hepes-KOH, pH 7.4, 0.6 *M* sorbitol buffer (HSB), and protein concentration was determined by the *DC* protein assay kit (Bio-Rad, Hercules, CA).

Mitochondrial import of frataxin

The plasmid pSP64T/FRDA was linearized with Bam*HI* and transcribed by using the Ribomax-SP6 kit (Promega, Madison, WI). Radiolabeled frataxin precursor protein was synthesized in reticulocyte lysate in the presence of ^{35}S Met-Cys Trans label by using the manufacturer's protocol (Promega).

Import reactions were performed as described (22) with some modifications. In brief, isolated mitochondria (150 μg of proteins) were incubated in HSB supplemented with 0.1 mg/ml BSA, 40 m*M* KOAc, 10 m*M* $Mg(OAc)_2$, 5 m*M* unlabeled methionine, 1 m*M* dithiothreitol (DTT), 4 m*M* adenosine triphosphate (ATP), 1 m*M* guanosine triphosphate (GTP), 20 m*M* phosphocreatine, and 0.2 mg/ml creatine kinase. Import was initiated by the addition of ^{35}S-Met–labeled precursor protein. After incubation at 28°C for various time periods (5–50 min), samples were treated on ice with 0.1 mg/ml trypsin for 35 min. To inactivate trypsin, samples were diluted with HSB containing 5 mg/ml soybean trypsin inhibitor, 100 U/ml trasylol, and 1 m*M* PMSF. Mitochondria were then sedimented (15,000 *g* for 10 min at 4°C) and washed with 10% trichloroacetic acid. Samples were analyzed with sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis followed by autoradiography. Bands corresponding to precursor and mature proteins were quantified by using densitometry and NIH J-image-analysis software.

Mitochondrial membrane potential ($\Delta\Psi$)

$\Delta\Psi$ was determined by both microscopy and flow cytometry. In brief, cell cultures were incubated (15 min at 37°C) in the dark with the lipophilic cation JC-1 probe (Invitrogen-Molecular Probes, Carlsbad, CA; 1.25 μg/ml) dissolved in growth medium. Cells were then washed with Hank's Balanced Salt Solution supplemented with $MgCl_2$ (1.2 m*M*) and $CaCl_2$ (0.8 m*M*) and viewed by using an Olympus IX-70 fluorescent microscope equipped with a high-resolution digital camera. Separate images of the same field using red and green filters were merged by using PictureFrame Software, version 1 (Olympus), and then analyzed by Soft Imaging System software (SIS), version 3.2 (Münster, Germany). The image was separated in the three fundamental colors (red, green, blue), and red and green images were analyzed with an adapted grey-level threshold to identify objects of interest. The total area of the objects was calculated and normalized to the area of the image.

For flow-cytometric analysis, JC-1–labeled cells were trypsinized, suspended in growth medium, washed twice with ice-cold Hank's Balanced Salt Solution, and analyzed by using a Becton Dickinson FACS analysis system equipped with a 488-nm argon laser for sample excitation. For positive control, cells were treated with valimomycin (10 μ*M*) for 15 min followed by JC-1 labeling. A shift from red to green fluorescence was considered indicative of mitochondrial membrane depolarization, which was expressed as the ratio of red and green mean fluorescence.

Autoradiographic measurement of labeling indices

Control or irradiated density-inhibited cultures were trypsinized, and the cells were suspended in growth medium containing [^{3}H]-thymidine at a concentration of 1 μCi/ml (specific activity, 20 Ci/mmol), seeded at low density in 30-mm dishes, and incubated at 37°C. At regular intervals, cells were fixed and submitted to autoradiographic examination, as described (7).

Cell-survival analysis

To measure repair of potentially lethal damage, clonogenic survival was assayed immediately after exposure of confluent, density-inhibited cultures to 4 Gy (3.3 Gy/min) or after various holding periods at 37°C (7). Survival values were corrected for the plating efficiency, which ranged from 15 to 20%.

Micronucleus assay

Radiation-induced DNA damage and its repair were assessed by measuring the frequency of micronucleus formation by the cytokinesis-block technique (2, 19). At least 1,000 cells were examined, and micronuclei in binucleated cells were analyzed. The frequency of micronucleus formation (r_0) was calculated as $r_0 = a/b$, where a is the total number of micronuclei scored, and b is the total of binucleated cells examined. The error associated with r_0 is given by the following formula: $\Delta r_0 = [(a/b)\,(1 - a/b)]^{1/2}$.

Statistical analysis

Student's *t* test and analysis of variance (ANOVA) were used to determine the significance of differences in the magnitude of measured end points before and after irradiation. Experiments were repeated at least twice, and standard error of the mean is indicated on the figures when greater than the size of the datum point symbol.

RESULTS

Effects of high-dose ionizing radiation on mitochondrial protein import

Most mitochondrial precursor proteins are imported post-translationally into mitochondria both *in vivo* and *in vitro*. The import process is mediated by translocases in the outer and inner membranes of mitochondria (21). Cellular exposure to radiation may cause damage to components of protein translocases, and this in turn may result in reduced/defective protein targeting/import. To investigate this possibility, we tested import of a matrix precursor protein into mitochondria isolated from irradiated confluent, density-inhibited normal human fibroblast cultures. This *in vitro* assay obviates the complexity that may arise from radiation-induced *in vivo* transcription/translational effects on cytoplasmically synthesized mitochondrial precursor proteins. As a prototype matrix protein, we chose the human protein frataxin because it follows the same import pathway as observed with other matrix proteins, and its import characteristics are well established in our laboratory (22). As in the case for many nuclear-encoded mitochondrial proteins, the frataxin precursor contains an N-terminal signal sequence

that is removed by a matrix-localized processing peptidase (MPP). Frataxin translocation into the matrix of isolated mitochondria was therefore assayed by the following criteria: (a) cleavage of the signal sequence by MPP, (b) protection of the mature polypeptide from digestion by exogenous proteases, and (c) co-sedimentation of imported molecules with mitochondria.

AG1522 normal human fibroblast cultures (95–98% in G_0/G_1) were exposed to a γ-ray dose of 4 Gy (3.3 Gy/min) that results in ~10% clonogenic survival. Cells were then incubated at 37°C for different periods (10 min, 1 h, and 12 h), harvested, and mitochondria isolated. ^{35}S-labeled frataxin precursor ("p") was synthesized in reticulocyte lysate, and its import into these mitochondria was tested over a period of 5–40 min. Frataxin import (as judged by the appearance of trypsin-protected mature ("m") molecules) into mitochondria isolated from cells held at 37°C for 10 min after irradiation was comparable to that observed with mitochondria from nonirradiated control cells (Fig. 1A). A significant decrease (threefold at 30 min by scanning densitometry) in import, however, was observed with mitochondria from cells held at 37°C for 1 h after exposure, suggesting that the processes that lead to defective import were not immediate, even though cells were exposed to a high dose of radiation (Fig. 1A). A slight attenuation in the import defect was observed with mitochondria isolated from cells held at 37°C for 12 h after exposure (Fig. 1A). Such an observation may imply that processes such as repair/elimination of damaged mitochondria, new mitochondrial biogenesis, and/or other recovery processes are slow in irradiated cells.

Effects of high-dose ionizing radiation on mitochondrial membrane potential

Translocation of precursor proteins across the inner membrane into the matrix is strictly dependent on a membrane potential ($\Delta\Psi$) across this membrane. Membrane potential (negative inside) perhaps produces an electrophoretic effect on positively charged presequences and thereby facilitates the initiation of translocation across the inner membrane (21). We therefore examined the effects of radiation on membrane potential and whether changes in $\Delta\Psi$ correlate with the radiation-induced changes in frataxin import. A significant decrease (~30%, $p < 0.01$) in $\Delta\Psi$ was noticed in cells exposed to 4 Gy and held in the density-inhibited state for 10 min or 1 h (Fig. 1B). It is interesting to note that we observed a significant import defect at the 1-h time point but not at the 10-min time point (Fig. 1A), even though the mitochondrial membrane potential was virtually identical at these two times (Fig. 1B). During the 12-h recovery period, mitochondrial $\Delta\Psi$ was restored close to the normal level (Fig. 1B), but the import defect continued to persist (Fig. 1A). Thus, the observed defects in frataxin import at 1-h and 12-h time points were not solely due to changes in $\Delta\Psi$. Such an effect could be due to damages in critical proteins that participate in the import process. In contrast to the import defect, chromosomal damage (as judged by micronuclei formation) (Fig. 1C) and potentially lethal damage (Fig. 1D) were found to be rapidly recovered (within 3 to 4 h) after exposure to 4 Gy. By 1.75 h after exposure, recovery from this damage was highly significant ($p \leq 0.001$). Collectively, the data in Fig. 1 thus suggest that recovery from the clastogenic effects of radiation may not be

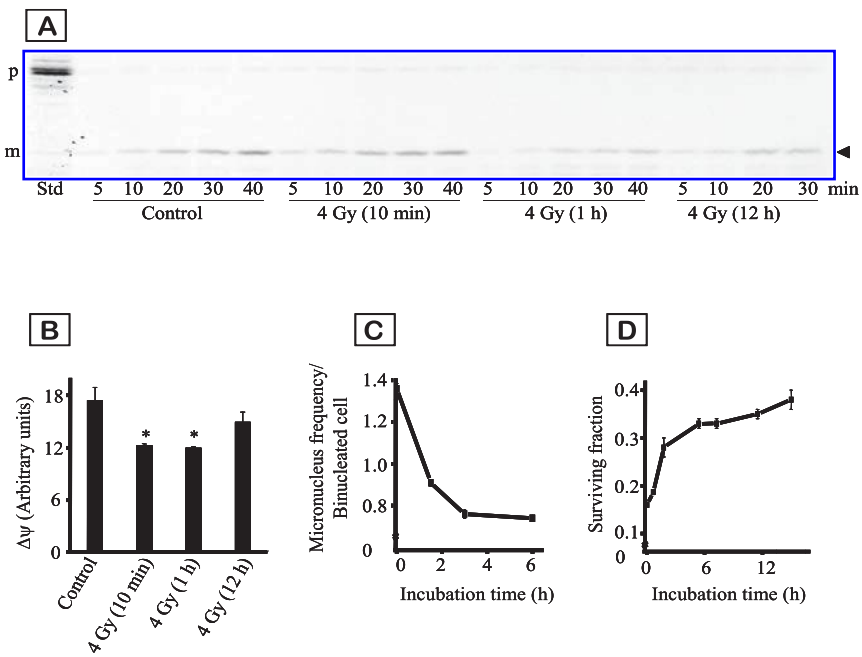

FIG. 1. Mitochondrial functions in control and high-dose γ-irradiated density-inhibited AG1522 fibroblasts. (**A**) Confluent cells were exposed to 0 or 4 Gy (3.3 Gy/min) and held at 37°C for various time periods (10 min, 1 h, 12 h). The import of frataxin into mitochondria isolated from these cells was evaluated. (**B**) Cells were exposed to γ-rays as in (**A**); at various time after exposure, cells were treated with JC-1 probe, harvested, and the mitochondrial membrane potential ($\Delta\Psi$) was determined by flow cytometry. Arbitrary units represent the ratio of mean red and green fluorescence. *$p \leq 0.01$. (**C**) Frequency of micronucleus formation in confluent cells exposed to γ-rays (4 Gy) and held in confluence at 37°C for various periods. The spontaneous micronucleus formation frequencies ranged from 0.005 to 0.03. (**D**) Clonogenic survival of confluent cells exposed to γ-rays (4 Gy) and held in confluence at 37°C for various periods. Cellular plating efficiencies ranged from 15 to 20%.

tightly linked to mitochondrial protein import and changes in mitochondrial membrane potential.

Mitochondrial import defects in irradiated density-inhibited cells are modulated by cell cycle–dependent effects

The cellular radiation response varies at different stages in the growth cycle (43, 47). Moreover, ionizing radiation as well as perturbations in the cellular capacity to generate ROS induces delays in G_1, S, and G_2 phases (28, 48). To investigate whether radiation-induced changes in mitochondrial functions are affected by movement of irradiated cells through the growth cycle, confluent density-inhibited AG1522 cell cultures were exposed to 0 or 4 Gy (3.3 Gy/min) and subcultured to a lower density within 10 min after irradiation. Progression into the cell cycle, frataxin import, and $\Delta\Psi$ were monitored at different times thereafter.

The cumulative labeling index data in Fig. 2A show that control cells began to enter S phase 18 h after subculture, and ~60% of the cells were in S phase by 24 h. Irradiated cells experienced a longer lag in G_1 before movement into S phase. As expected (7), at 50% of the maximum labeling indices, irradiated cells were delayed by ~12 h in their progression into S phase (Fig. 2A). For import experiments, care was taken to ensure that after subculture, control and irradiated cells were at similar stages of the cell cycle. Specifically, irradiated cells were harvested 4 or 12 h later than control cells to examine effects in mid-G_1 and S phases, respectively.

Experiments led to the following four important observations. First, the efficiency of import and membrane potential in nonirradiated control cells were found to be cell cycle dependent. Frataxin import was more efficient (1.5-fold greater) in G_1 (Fig. 2B, panel a) compared with that observed in S phase (Fig. 2B, panel c). Likewise, the mitochondrial membrane potential in control cells was stronger in mid G_1 than in late G_1 or S phase ($p \leq 0.001$) (Fig. 2C). Second, no defect in mitochondrial import was observed when irradiated density-inhibited cells were subcultured and allowed to reach mid to late G_1 phase (Fig. 2B, compare panels a and b). This is in sharp contrast with the significant defects observed with mitochondria isolated from irradiated cells that were maintained in confluent, density-inhibited G_0/G_1 phase (Fig. 1A, panels depicting cells exposed to 4 Gy and maintained in quiescence for 1 or 12 h). Third, frataxin import into mitochondria isolated from irradiated cells that progressed to S phase (Fig. 2B, panel d) was reduced by 2.5-fold or 20% when compared, respectively, with irradiated cells that progressed to G_1 phase (Fig. 2B, panel b) or nonirradiated cells at a similar position in S phase (Fig. 2B, panel c). Fourth, although $\Delta\Psi$ appears to vary in a cell cycle–dependent manner in control cells (Fig. 2C), it does not appear to be a key determining factor in changes in mitochondrial import efficiency observed in irradiated cells that were allowed to progress in the cell cycle (Fig. 2B). Although $\Delta\Psi$ was decreased in G_1 and S phase (Fig. 2C), import was differentially modulated at these phases, being apparently unaffected in G_1 and decreased in S (Fig. 2B, panels b and d). Together, these results suggest that mitochondrial import and membrane potential in control cells are regulated in a cell cycle–dependent manner. Furthermore,

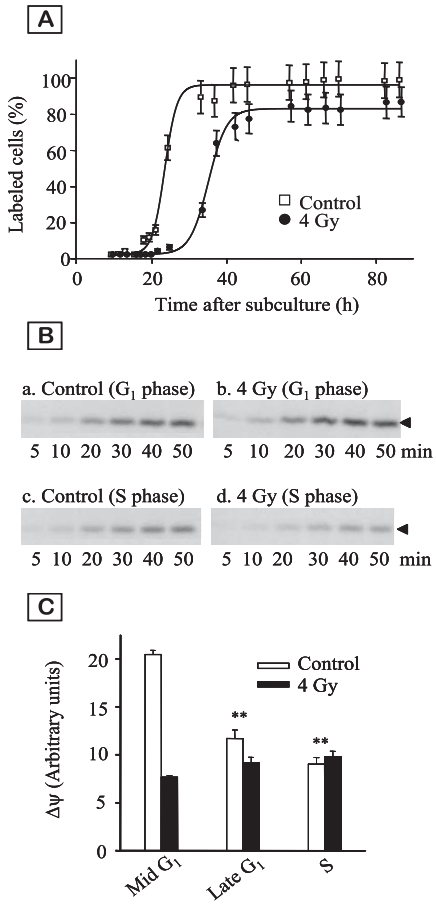

FIG. 2. Mitochondrial functions in control and high-dose γ-irradiated (4 Gy) density-inhibited AG1522 fibroblasts that were stimulated to proliferate by subculture to a lower density. (**A**) Progression from G_0/G_1 to S phase of the cell cycle as a function of time after release from the confluent state of control or irradiated cells. Cell cultures were trypsinized and seeded at a lower density in growth medium containing 1 μCi/ml ^{3}H-thymidine. The fraction of labeled cells was determined by autoradiography. (**B**) Frataxin import in mitochondria isolated from subcultured cells. Subcultured control and irradiated cells were harvested for analyses when they were at similar stage in the cell cycle. **a**: Control cells harvested at 8 h. **b**: Irradiated cells harvested at 12 h. **c**: Control cells harvested at 24 h. **d**: Irradiated cells harvested at 36 h. (**C**) Flow-cytometric measurement of mitochondrial membrane potential in subcultured cells labeled with JC-1 probe. Control and irradiated cells were harvested at 8 or 12 h, respectively, for analysis in mid G_1; 18 or 26 h, respectively, for analyses in late G_1; and 24 or 36 h, respectively, for analyses in S phase. $^{**}p \leq 0.001$.

whereas modulation of import in irradiated G_0/G_1 cells that have been stimulated to proliferate is not manifested at the G_1 phase (Fig. 2B, panel b), cellular events in S phase significantly modulate this effect (Fig. 2B, panel c). Such outcomes, which occur long after radiation exposure, may have resulted from direct effects of γ-rays on mitochondria or from effects in other organelles that altered mitochondrial function.

Exposure of human fibroblasts to low-dose/low-dose-rate ionizing radiation enhances the kinetics of mitochondrial import of frataxin

The biologic effects and health risks associated with exposure to low doses of ionizing radiation remain ambiguous (11) and are currently the subject of intense debate (30, 34, 38, 39). Generally, radiation-induced biologic effects are assumed to be strictly proportional to dose, however low it may be. Compared with high-dose-rate effects, low dose rates are thought to attenuate the severity of radiation effects by about a factor of two (10, 32). To investigate low-dose effects on mitochondrial function, frataxin import was investigated in control and low-dose/low-dose-rate exposed AG1522 fibroblasts. The representative import kinetics data in Fig. 3A indicate an enhancement of import (45% at 20 min) in mitochondria isolated from density-inhibited cells harvested immediately after exposure to 10 cGy (0.2 cGy/h). This is in contrast to the decrease in import observed in mitochondria from quiescent cells exposed to 4 Gy (3.3 Gy/min) (Fig. 1A). These data are inconsistent with a strict linearity of the dose–response relation (10). They show that biologic events induced by low- and high-dose radiation exposures differentially modulate mitochondrial import.

Similar to import (Fig. 3A), *in situ* $\Delta\Psi$ measurements (Fig. 3B) performed shortly after exposure of AG1522 cells to low-dose/low-dose-rate γ-rays (10 cGy, 0.2 cGy/h) showed an increase (2.5-fold) relative to control. In contrast, in cells exposed to 4 Gy (3.3 Gy/min), $\Delta\Psi$ was decreased (Fig. 1B).

DISCUSSION

Elucidation of the mechanisms underlying the long-term effects of ionizing radiation is currently under intensive investigation (9, 16). Oxidative metabolism has been shown to modulate both high- and low-dose radiation effects (6, 18). It has been singled out as a critical factor in the expression of radiation-induced degenerative diseases (46, 49). Chronic excess levels of ROS, which result from induced perturbation of metabolic reactions in irradiated cells, may cause cellular damage through specific signaling events and/or indiscriminate and cumulative effects. Most estimates suggest that the majority of intracellular ROS production is derived from energy generation in the mitochondria (1). How mitochondria respond to ionizing radiation is therefore critical for understanding the mechanism of radiation-induced signaling processes.

Protein import is essential for mitochondrial biogenesis, and import of most precursor proteins into this organelle requires membrane potential. We therefore examined changes in mitochondrial protein import and membrane potential in density-inhibited γ-irradiated cells that were maintained in the same quiescent state after exposure or were allowed to proliferate by subculturing to a lower density. Specifically, we compared effects due to high- or low-dose radiation. Our data show that the effects of low dose on mitochondrial function cannot be predicted from effects at high dose. Whereas import of frataxin, a protein located in the mitochondrial matrix, was decreased in quiescent cells exposed to high-dose γ-rays (Fig. 1A), it was increased in low-dose/low-dose-rate ir-

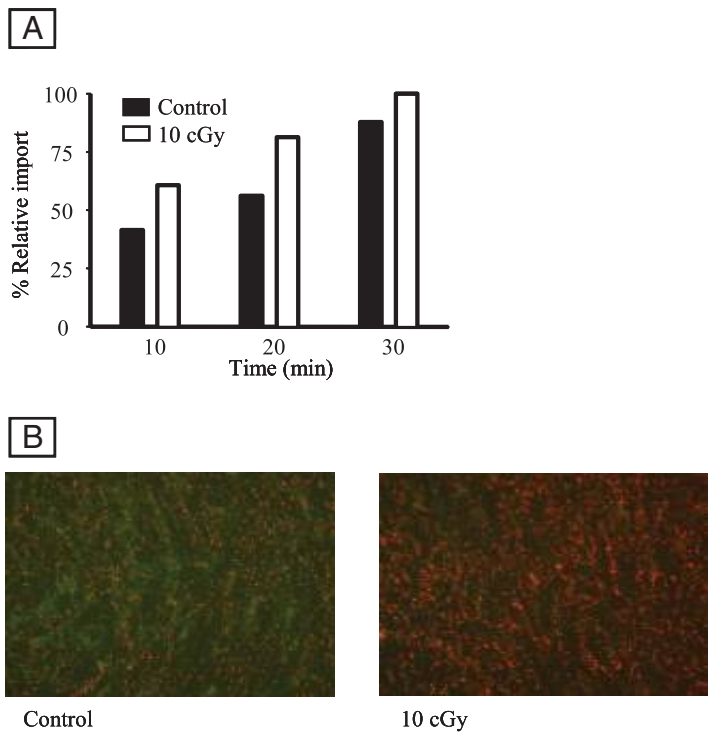

FIG. 3. Mitochondrial functions in control and low-dose/low-dose-rate γ-irradiated confluent, density-inhibited AG1522 fibroblasts. (**A**) Cells cultures were exposed to 0 or 10 cGy (0.2 cGy/h). Mitochondria were isolated immediately after the exposure, and the kinetics of frataxin import was determined. After gel electrophoresis and autoradiography, bands were quantified by scanning densitometry. Import at the 30-min time point in mitochondria from irradiated cells was considered to be 100%. (**B**) Cell cultures were exposed to γ-rays as in (**A**), and mitochondrial membrane potential in JC-1–labeled cells was determined by fluorescent microscopy. A shift from red to green fluorescence is indicative of membrane depolarization.

radiated cells (Fig. 3A). The radiation-induced changes in mitochondrial membrane potential of quiescent cells (Figs. 1B and 3B) mirrored the changes in import at high- and low-dose exposures (Figs. 1B and 3B). These data therefore argue against the currently assumed linearity of the dose–response relation (10).

Although mitochondrial protein import and $\Delta\Psi$ have been shown to be tightly linked, changes in $\Delta\Psi$ alone cannot explain radiation-induced changes in import efficiency/kinetics. For example, $\Delta\Psi$ was significantly and rapidly decreased when density-inhibited cells were exposed to a high dose (4 Gy) and held in the confluent state for 10 min, and remained unchanged for $\geq$1 h (Fig. 1B). The import defect, however, was observed at the 1-h time point but not at the 10-min time point (Fig. 1A), even though $\Delta\Psi$ was virtually the same at these two times. We propose that $\Delta\Psi$ is not the only factor that determines import efficiency of mitochondria in irradiated G_0/G_1 cells.

Another major finding of this study is that in control nonirradiated cells, $\Delta\Psi$ and import are regulated in a cell cycle–dependent manner, with $\Delta\Psi$ being lower in late G_1 and *S* than in mid G_1 (Fig. 2C) and import being lower in *S* than in G_1 (Fig. 2B). Furthermore, events associated with *S* phase, but not G_1, appear to affect mitochondrial import function significantly in G_0/G_1 irradiated cells that have been stimulated to proliferate (Fig. 2B). Changes in mitochondrial morphology and DNA distribution are known to occur in a cell cycle-dependent manner (31). It would be interesting to determine whether these changes also affect $\Delta\Psi$ and protein import and whether overall mitochondrial alterations contribute to radiation-induced checkpoints.

The exact mechanisms underlying repair of potentially lethal damage (Fig. 1D) remain unclear (28). Our data suggest that the modulation of mitochondrial import (Fig. 1A) by high-dose radiation (4 Gy) is not tightly linked to the cellular capacity to repair such damage. The situation for low-dose radiation (10 cGy), however, may be different, and protective/adapting effects induced by low-dose radiation (4, 5, 37) may be more closely related to increased mitochondrial import and membrane potential observed at these doses (Fig. 3A and B). It is attractive to speculate that mitochondria-mediated events are involved in signaling the expression of adaptive responses. This is supported by recent evidence that induction of the redox-sensitive transcription factor nuclear factor (NF)κB regulates expression of manganese superoxide dismutase, a mitochondrial protein that regulates the expression of genes that participate in radiation-induced adaptive responses (25). We are currently investigating whether the decrease in import (Figs. 1A and 2B) in cells exposed to high-dose γ-rays is attenuated when they are preexposed to a prolonged 10-cGy adapting dose. We also are examining the correlation between the pattern of changes in mitochondrial functions and levels of ROS in irradiated cells. Such information would be further enlightening of potential relations between low-dose–induced protective effects, mitochondrial function, and oxidative stress.

To examine the role of mitochondria in the radiation response, cells have often been depleted of mitochondrial DNA (rho^0 cells). Although these studies have been informative, many cellular functions are likely to be impaired in rho^0 cells, including the expression of nuclear genes (50). Such effects would complicate the interpretation of results. In contrast, this study outlines a strategy to examine mitochondrial function in irradiated cells, which do not suffer from nonspecific secondary effects because of prior deletion of organellar DNA. In summary, this study leads to new approaches to examine further the mechanisms underlying high- and low-dose effects. It demonstrates the sensitivity of mitochondrial protein import and membrane potential in characterizing radiation effects related to oxidative metabolism.

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ABBREVIATIONS

$\Delta\Psi$, Mitochondrial membrane potential; HSB, Hepes/sorbitol buffer; MPP, matrix-localized processing peptidase; ROS, reactive oxygen species.

REFERENCES

1. Andreyev AY, Kushnareva YE, and Starkov AA. Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc)* 70: 200–214, 2005.
2. Azzam EI, de Toledo SM, and Little JB. Direct evidence for the participation of gap-junction mediated intercellular communication in the transmission of damage signals from alpha-particle irradiated to non-irradiated cells. *Proc Natl Acad Sci U S A* 98: 473–478, 2001.
3. Azzam EI, de Toledo SM, and Little JB. Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. *Oncogene* 22: 7050–7057, 2003.
4. Azzam EI, de Toledo SM, Raaphorst GP, and Mitchel RE. Réponse adaptative au rayonnement ionisant des fibroblastes de peau humaine: Augmentation de la vitesse de réparation de l'ADN et variation de l'expression des gènes. *J Chim Phys* 91: 931–936, 1994.
5. Azzam EI, de Toledo SM, Raaphorst GP, and Mitchel RE. Low-dose ionizing radiation decreases the frequency of neoplastic transformation to a level below the spontaneous rate in C3H 10T1/2 cells. *Radiat Res* 146: 369–373, 1996.
6. Azzam EI, de Toledo SM, Spitz DR, and Little JB. Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from alpha-

particle-irradiated normal human fibroblast cultures. *Cancer Res* 62: 5436–5442, 2002.
7. Azzam EI, de Toledo SM, Waker AJ, and Little JB. High and low fluences of alpha-particles induce a G_1 checkpoint in human diploid fibroblasts. *Cancer Res* 60: 2623–2631, 2000.
8. Azzam EI and Little JB. The radiation-induced bystander effect: Evidence and significance. *Hum Exp Toxicol* 23: 61–65, 2004.
9. Bedford JS and Dewey WC. Radiation Research Society, 1952–2002: Historical and current highlights in radiation biology: Has anything important been learned by irradiating cells? *Radiat Res* 158: 251–291, 2002.
10. *BEIR-VII Health risks from exposure to low levels of ionizing radiation.* Washington, DC: National Research Council of the National Academies, 2005.
11. Bonner WM. Low-dose radiation: Thresholds, bystander effects, and adaptive responses. *Proc Natl Acad Sci U S A* 100: 4973–4975, 2003.
12. Boyce RP and Howard-Flanders P. Genetic control of DNA breakdown and repair in *E. coli* K-12 treated with mitomycin C or ultraviolet light. *Z Vererbungsl* 95: 345–350, 1964.
13. Brooks AL. Paradigm shifts in radiation biology: Their impact on intervention for radiation-induced disease. *Radiat Res* 164: 454–461, 2005.
14. Chance B, Sies H, and Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59: 527–605, 1979.
15. Chang WP and Little JB. Delayed reproductive death as a dominant phenotype in cell clones surviving X-irradiation. *Carcinogenesis* 13: 923–928, 1992.
16. Coleman CN, Blakely WF, Fike JR, MacVittie TJ, Metting NF, Mitchell JB, Moulder JE, Preston RJ, Seed TM, Stone HB, Tofilon PJ, and Wong RS. Molecular and cellular biology of moderate-dose (1–10 Gy) radiation and potential mechanisms of radiation protection: Report of a workshop at Bethesda, Maryland, December 17–18, 2001. *Radiat Res* 159: 812–834, 2003.
17. Daniel PT, Schulze-Osthoff K, Belka C, and Guner D. Guardians of cell death: The Bcl-2 family proteins. *Essays Biochem* 39: 73–88, 2003.
18. Feinendegen LE. Reactive oxygen species in cell responses to toxic agents. *Hum Exp Toxicol* 21: 85–90, 2002.
19. Fenech M and Morley AA. Measurement of micronuclei in lymphocytes. *Mutat Res* 147: 29–36, 1985.
20. Finkel T and Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239–247, 2000.
21. Gordon DM, Dancis A, and Pain D. Mechanisms of mitochondrial protein import. *Essays Biochem* 36: 61–73, 2000.
22. Gordon DM, Shi Q, Dancis A, and Pain D. Maturation of frataxin within mammalian and yeast mitochondria: One-step processing by matrix processing peptidase. *Hum Mol Genet* 8: 2255–2262, 1999.
23. Gorgojo L and Little JB. Expression of lethal mutations in progeny of irradiated mammalian cells. *Int J Radiat Biol* 55: 619–630, 1989.
24. Green DR. Apoptotic pathways: Ten minutes to dead. *Cell* 121: 671–674, 2005.
25. Guo G, Yan-Sanders Y, Lyn-Cook BD, Wang T, Tamae D, Ogi J, Khaletskiy A, Li Z, Weydert C, Longmate JA, Huang TT, Spitz DR, Oberley LW, and Li JJ. Manganese superoxide dismutase-mediated gene expression in radiation-induced adaptive responses. *Mol Cell Biol* 23: 2362–2378, 2003.
26. Jacobson MD, Weil M, and Raff MC. Programmed cell death in animal development. *Cell* 88: 347–354, 1997.
27. Kennedy AR, Cairns J, and Little JB. Timing of the steps in transformation of C3H 10T1/2 cells by X-irradiation. *Nature* 307: 85–86, 1984.
28. Little JB. Failla Memorial Lecture: Changing views of cellular radiosensitivity. *Radiat Res* 140: 299–311, 1994.
29. Little JB. Genomic instability and bystander effects: A historical perspective. *Oncogene* 22: 6978–6987, 2003.
30. Little MP and Muirhead CR. Derivation of low-dose extrapolation factors from analysis of curvature in the cancer incidence dose response in Japanese atomic bomb survivors. *Int J Radiat Biol* 76: 939–953, 2000.
31. Ma J and Wehrli FW. Method of image-based measurement of the reversible and irreversible contribution to the transverse-relaxation rate. *J Magn Reson Series B* 111: 61–69, 1996.
32. Mettler FA, Sinclair WK, Anspaugh L, Edington C, Harley JH, Ricks RC, Selby PB, Webster EW, and Wyckoff HO. The 1986 and 1988 UNSCEAR (United Nations Scientific Committee on the Effects of Atomic Radiation) reports: Findings and implications. *Health Phys* 58: 241–250, 1990.
33. Mirzayans R, Scott A, Cameron M, and Murray D. Induction of accelerated senescence by gamma radiation in human solid tumor-derived cell lines expressing wild-type TP53. *Radiat Res* 163: 53–62, 2005.
34. Mitchel RE, Jackson JS, McCann RA, and Boreham DR. The adaptive response modifies latency for radiation-induced myeloid leukemia in CBA/H mice. *Radiat Res* 152: 273–279, 1999.
35. Murakami H, Pain D, and Blobel G. 70-kD Heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. *J Cell Biol* 107: 2051–2057, 1988.
36. Nelson KK and Melendez JA. Mitochondrial redox control of matrix metalloproteinases. *Free Radic Biol Med* 37: 768–784, 2004.
37. Olivieri G, Bodycote J, and Wolff S. Adaptive response of human lymphocytes to low concentrations of radioactive thymidine. *Science* 223: 594–597, 1984.
38. Preston DL, Pierce DA, Shimizu Y, Ron E, and Mabuchi K. Dose response and temporal patterns of radiation-associated solid cancer risks. *Health Phys* 85: 43–46, 2003.
39. Preston RJ. The LNT model is the best we can do—today. *J Radiol Prot* 23: 263–268, 2003.
40. Schollnberger H, Mitchel RE, Azzam EI, Crawford-Brown DJ, and Hofmann W. Explanation of protective effects of low doses of gamma-radiation with a mechanistic radiobiological model. *Int J Radiat Biol* 78: 1159–1173, 2002.
41. Scott BR. A biological-based model that links genomic instability, bystander effects, and adaptive response. *Mutat Res* 568: 129–143, 2004.

42. Setlow RB and Carrier WL. The disappearance of thymine dimers from DNA: An error-correcting mechanism. *Proc Natl Acad Sci U S A* 51: 226–231, 1964.
43. Sinclair WK and Morton RA. X-ray sensitivity during the cell generation cycle of cultured Chinese hamster cells. *Radiat Res* 29: 450–474, 1966.
44. Singh A and Singh H. Time-scale and nature of radiation-biological damage: Approaches to radiation protection and post-irradiation therapy. *Prog Biophys Mol Biol* 39: 69–107, 1982.
45. Skulachev VP. Mitochondrial physiology and pathology; concepts of programmed death of organelles, cells and organisms. *Mol Aspects Med* 20: 139–184, 1999.
46. Spitz DR, Azzam EI, Li JJ, and Gius D. Metabolic oxidation/reduction reactions and cellular responses to ionizing radiation: A unifying concept in stress response biology. *Cancer Metastasis Rev* 23: 311–322, 2004.
47. Terasima R and Tolmach LJ. X-ray sensitivity and DNA synthesis in synchronous populations of HeLa cells. *Science* 140: 490–492, 1963.
48. Venkatachalam P, de Toledo SM, and Azzam EI. Flavin-containing oxidases regulate progression from G_1 to S phase of the cell cycle in normal human fibroblasts. *Radiat Phys Chem* 72: 315–321, 2004.
49. Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: A dawn for evolutionary medicine. *Annu Rev Genet* 39: 359–407, 2005.
50. Wang H and Morais R. Up-regulation of nuclear genes in response to inhibition of mitochondrial DNA expression in chicken cells. *Biochim Biophys Acta* 1352: 325–334, 1997.
51. Wolff S. Failla Memorial Lecture: Is radiation all bad? The search for adaptation. *Radiat Res* 131: 117–123, 1992.
52. Zirkle RE and Bloom W. Irradiation of parts of individual cells. *Science* 117: 487–493, 1953.

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2. Edouard I. Azzam, Jean-Paul Jay-Gerin, Debkumar Pain. 2011. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. *Cancer Letters* . [CrossRef]
3. Govindasamy Kanimozhi, Nagarajan Rajendra Prasad, Samivel Ramachandran, Kodukkur Viswanathan Pugalendi. 2011. Umbelliferone modulates gamma-radiation induced reactive oxygen species generation and subsequent oxidative damage in human blood lymphocytes. *European Journal of Pharmacology* . [CrossRef]
4. Narongchai Autsavapromporn, Sonia M. de Toledo, John B. Little, Jean-Paul Jay-Gerin, Andrew L. Harris, Edouard I. Azzam. 2011. The Role of Gap Junction Communication and Oxidative Stress in the Propagation of Toxic Effects among High-Dose #-Particle-Irradiated Human Cells. *Radiation Research* **175**:3, 347-357. [CrossRef]
5. Nukhet Aykin-Burns, Benjamin G. Slane, Annie T. Y. Liu, Kjerstin M. Owens, Malinda S. O'Malley, Brian J. Smith, Frederick E. Domann, Douglas R. Spitz. 2011. Sensitivity to Low-Dose/Low-LET Ionizing Radiation in Mammalian Cells Harboring Mutations in Succinate Dehydrogenase Subunit C is Governed by Mitochondria-Derived Reactive Oxygen Species. *Radiation Research* **175**:2, 150-158. [CrossRef]
6. Massimo Pinto, Edouard I. Azzam, Roger W. Howell. 2010. Investigation of Adaptive Responses in Bystander Cells in 3D Cultures Containing Tritium-Labeled and Unlabeled Normal Human Fibroblasts. *Radiation Research* **174**:2, 216-227. [CrossRef]
7. Binod Kumar, Amit Kumar, B. N. Pandey, K. P. Mishra, Banasri Hazra. 2009. Role of mitochondrial oxidative stress in the apoptosis induced by diospyrin diethylether in human breast carcinoma (MCF-7) cells. *Molecular and Cellular Biochemistry* **320**:1-2, 185-195. [CrossRef]
8. K M Ahmed, M Fan, D Nantajit, N Cao, J J Li. 2008. Cyclin D1 in low-dose radiation-induced adaptive resistance. *Oncogene* **27**:53, 6738-6748. [CrossRef]
9. P Venkatachalam, S M de Toledo, B N Pandey, L A Tephly, A B Carter, J B Little, D R Spitz, E I Azzam. 2008. Regulation of normal cell cycle progression by flavin-containing oxidases. *Oncogene* **27**:1, 20-31. [CrossRef]
10. Daniel Sirk, Ziping Zhu, Jehangir S. Wadia, Natalya Shulyakova, Nam Phan, Jamie Fong, Linda R. Mills. 2007. Chronic exposure to sub-lethal beta-amyloid (A#) inhibits the import of nuclear-encoded proteins to mitochondria in differentiated PC12 cells*. *Journal of Neurochemistry* **103**:5, 1989-2003. [CrossRef]
11. Sonia M. de Toledo, Nesrin Asaad, Perumal Venkatachalam, Ling Li, Roger W. Howell, Douglas R. Spitz, Edouard I. Azzam. 2006. Adaptive Responses to Low-Dose/Low-Dose-Rate # Rays in Normal Human Fibroblasts: The Role of Growth Architecture and Oxidative Metabolism. *Radiation Research* **166**:6, 849-857. [CrossRef]