A Role for Mitochondrial Dysfunction in Perpetuating Radiation-Induced Genomic Instability

Grace J. Kim,1,2 Gary M. Fiskum,3 and William F. Morgan2,4

1Graduate Program in Molecular and Cell Biology, Biomedical Research Facility, 2Radiation Oncology Research Laboratory, Department of Radiation Oncology, 3Department of Anesthesiology, and 4Marlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland

Abstract

Radiation-induced genomic instability (RIGI) manifests as a heritable increased rate of genetic alterations in the progeny of irradiated cells generations after the initial insult. The progeny can show an increased frequency of chromosomal translocations, deletions, mutations, micronuclei, and decreased plating efficiency. What perpetuates RIGI is unclear; however, persistently increased levels of reactive oxygen species (ROS) are frequently associated with genomically unstable clones. Furthermore, addition of free radical scavengers (e.g., DMSO, glycerol, and cationic thiol cysteamine) reduces the incidence of instability after irradiation, implicating a ROS-mediated role in RIGI induction. Because mitochondria are a major natural cellular source of ROS, we tested the hypothesis that mitochondrial dysfunction has a role in maintaining the elevated ROS levels in our irradiated, genetically unstable GM10115 Chinese hamster ovary cells. Amplex Red fluorometry measurements indicate that the relative contribution of uncoupler-sensitive mitochondrial hydrogen peroxide production to total cellular hydrogen peroxide generation is greater in unstable cells. Measurements of mitochondrial DNA levels and cell cytometric fluorescent measurements of Mitotracker Green FM indicate that differences in mitochondrial ROS production are not due to varying mitochondrial locations, deletions, mutations, micronuclei, and decreased respiration rates may explain the increased levels of cellular ROS. These studies show that mitochondria from unstable cells are abnormal and likely contribute to the persistent oxidative stress in the unstable clones. (Cancer Res 2006; 66(21): 10377-83)

Introduction

Accumulating evidence suggests that radiation-induced genomic instability (RIGI) is a nontargeted phenomenon triggered by radiation that may initiate and likely contribute to radiation carcinogenesis (1). RIGI is defined as an increased heritable frequency of nuclear genetic alterations in the progeny of the irradiated cell long after the initial radiation exposure. In RIGI, the progeny of irradiated cells display changes, such as aneuploidy, micronucleus formation, sister chromatid exchanges, gene mutations, amplifications, reduced plating efficiency, and chromosomal destabilization (2). Genomic instability is believed to play a role in promoting tumorigenesis through genome destabilization resulting in the high frequencies of genomic changes seen in most cancers (3).

The frequency of RIGI initiation exceeds the frequency of nuclear gene mutation at the same radiation dose; thus, a single gene mutation cannot be the initiating event (4). DNA damage caused by direct radiation cannot explain the persistence of these delayed phenotypes in the progeny of irradiated cells that themselves saw no radiation (5). There is growing evidence that oxidative stress has a role in instability initiation and perpetuation (6). High levels of free radicals as well as products of oxidative stress, including oxidative base damage, have been described in radiation-induced chromosomally unstable cell lines by different investigators. In fact, in unstable cells derived from our GM10115 system, Limoli et al. found increased levels of lipid peroxidation products (7–9). Chronic hydrogen peroxide (H2O2) or glucose oxidase administration induces instability (10, 11). Likewise, free radical scavenger treatment and hypoxic conditions reduce the incidence of delayed genetic instability after irradiation (12, 13), implicating a role for reactive oxygen species (ROS) in RIGI induction. In our unstable cell lines, we find increased levels of ROS many generations after the radiation insult (9). Ionizing radiation transiently increases cellular ROS levels through water hydrolysis, but these radicals are extremely short lived (e.g., the hydroxyl radical has a lifetime of ~10^-6 seconds; ref. 14). Thus, ROS must be continuously produced as we find persistently increased levels in the unstable clones for prolonged periods after the initial radiation insult (8, 9).

Several cellular sources can generate ROS: peroxisomes, plasma membrane proteins, such as NAPDH oxidase, cytosolic enzymatic reactions, and mitochondria. Mitochondria contribute to ~90% of total cellular ROS levels (15) where normally 1% to 5% of the oxygen it consumes is converted into superoxide anions and other ROS (16). Damaged mitochondria can produce even more free radicals from an impaired electron transport chain (17), increasing the potential for chromosomal abnormalities (18). Some of the consequences of dysfunctional respiration are highlighted in a study that observed a point mutation in the mitochondrial DNA (mtDNA) gene MTATP6 causing deficient mitochondrial respiratory activity and promotion of carcinogenesis through inhibition of apoptosis (19).

Preliminary studies examining mitochondria from radiation-induced genetically unstable cells showed abnormal membrane potential, the driving force for ATP synthesis (8). We examined mitochondrial function in genetically unstable clones to test the hypothesis that dysfunctional mitochondria contribute to the persistently increased ROS levels. Our findings support this hypothesis and provide further evidence for a role for mitochondrial dysfunction in RIGI.
Materials and Methods

Cell culture. Chinese hamster ovary (CHO) cells, GM10115 (Human Genetic Mutant Cell Repository, Camden, NJ), which contain one copy of human chromosome 4 in a background of 20 to 24 CHO chromosomes, were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units penicillin, 100 μg/mL streptomycin, 2 mM/L glutamine, and 0.2 mM/L L-proline. Cultures were maintained in 5% CO₂ in air at 34°C.

Stable and unstable colonies were isolated from irradiated parental GM10115 cells by isolating and clonally expanding a surviving irradiated cell (20-22). Unstable clones were identified through cytogenetic analysis of metaphase chromosomes using fluorescence in situ hybridization for human chromosome 4. At a dose of 1 Gy, ~3% of clones analyzed are genetically unstable (5). An unstable clone contains at least three metaphase subpopulations with unique rearrangements of the human chromosome. These rearranged subpopulations consist of ≥5% of the 20 metaphases scored. Two unstable clones, LS12 and Fe10-3, isolated from GM10115 cells irradiated with 10 Gy of X-rays or iron ions, respectively, were used in this investigation. LS12 and Fe10-3 showed 13 and 18 unique rearrangements subpopulations with unique rearrangements of the human chromosome.

To determine levels of mitochondria within each clone (28), a suspension of 5 × 10⁶ cells/mL in PBS was loaded with freshly prepared 5 μM/L Mitotracker Green FM (Molecular Probes) binds to mitochondrial membrane lipids regardless of mitochondrial respiratory activity, p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP, Sigma), was used to probe for the mitochondrial contribution to cellular H₂O₂ production (24). The rate of uninhibited ROS production of intact cells (5 × 10⁶ cells/mL) in NaCl buffer (140 mM/L NaCl, 3 mM/L KCl, 0.4 mM/L KH₃PO₄, 20 mM/L HEPES, 5 mM/L NaHCO₃, 5 mM/L glucose, 1 mM/L MgCl₂) was measured at 30 minutes, spun down, and rinsed once with PBS. Dead cells were excluded from analysis using 5 mM/L propidium iodide, which is unable to stain live cells with intact membranes. Fluorescence intensity representing ROS levels of live cells was measured using flow cytometry using a Becton Dickinson FACScan flow cytometer (Franklin Lakes, NJ).

H₂O₂ production. The generation of H₂O₂ was measured using 1 mM/L Amplex Red ( Molecular Probes) in DMSO in the presence of 40 units/mL superoxide dismutase (SOD) and 10 units/mL horseradish peroxidase (HRP; Sigma, St. Louis, MO). Amplex Red reacts with H₂O₂ producing a fluorescent signal, measured at excitation and emission wavelengths of 563 and 587 nm, respectively, using a fluorescence spectrophotometer with a temperature-regulated chamber equipped with constant stirring. H₂O₂ is generated by the reaction between mitochondrial-produced superoxide radical and SOD. SOD was also used to prevent auto-oxidation of Amplex Red that could interfere with H₂O₂ measurements. The Amplex Red signal was calibrated by sequential additions of fresh H₂O₂. The mitochondrial respiratory uncoupler, p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP, Sigma), was used to probe for the mitochondrial contribution to cellular H₂O₂ production (24). The rate of uninhibited ROS production of intact cells (5 × 10⁶ cells/mL) in NaCl buffer (140 mM/L NaCl, 3 mM/L KCl, 0.4 mM/L KH₃PO₄, 20 mM/L HEPES, 5 mM/L NaHCO₃, 5 mM/L glucose, 1 mM/L MgCl₂) was measured at 34°C with constant stirring. FCCP (10 μM/L) was then added causing a reduction in the rate of H₂O₂ generation. The difference in the rate of H₂O₂ ± FCCP is defined as the minimum H₂O₂ contribution of mitochondria to total cellular H₂O₂ production.

Mitochondrial mass. The mitochondrial probe Mitotracker Green FM ( Molecular Probes) binds to mitochondrial membrane lipids regardless of mitochondrial membrane potential or oxidant status (25). Although nonyl acridine orange has been used to determine mitochondrial mass (26), binding may be influenced by altered mitochondrial respiratory states (27). To determine levels of mitochondria within each clone (28), a suspension of 1 × 10⁷ cells/mL in PBS was loaded with 250 mM/L Mitotracker Green FM in DMSO for 30 minutes at 34°C. Flow cytometric fluorescence measurements were made with excitation at 490 nm and emission at 516 nm using a Becton Dickinson FACScan flow cytometer. Amounts were determined by comparing the means of the fluorescent signal.

mtDNA quantification. Total cellular DNA was isolated using GenElute Mammalian genomic DNA kit (Sigma). Total cellular DNA (20 μg) was digested with 5 units/μg Kpn1, which linearizes all mtDNA because CHO mtDNA has a single Kpn1 restriction site (29). Fragments were run on a 0.7% agarose gel and then transferred and fixed to a positively charged nylon membrane. The membrane was hybridized with [32P]DAP-labeled cytochrome c oxidase subunit 2 and subunit 3 (cytochrome oxidase (COX) 2 and COX3) genes to detect levels of mtDNA. mtDNA levels were normalized to β-actin DNA. Membranes were washed for 2 hours with 2% SSC, 0.1% SDS buffer, 2 hours with 0.1% SSC, 1% SDS buffer, and then 2 hours with 0.1% SSC buffer at 65°C. Membranes were analyzed using phosphoimaging (Bio-Rad Personal Molecular Imaging FX, Hercules, CA) and band intensities were quantified using Quantity One software (Bio-Rad).

Digitonin-permeabilized respiration. Mitochondrial oxygen consumption within cells was measured using a Clark-type oxygen electrode in a thermostatically controlled chamber. Cells were trypsinized, rinsed with PBS, and resuspended in 100 μL of a sodium chloride buffer (140 mM/L NaCl, 3 mM/L KCl, 0.4 mM/L KH₃PO₄, 20 mM/L HEPES, 5 mM/L NaHCO₃, 5 mM/L glucose, 1 mM/L MgCl₂) and then brought to a final concentration of 2 × 10⁵ cells/mL in a potassium buffer [125 mM/L KCl, 2 mM/L KH₂PO₄, 10 mM/L HEPES, 1 mM/L MgCl₂ (pH 7.0)] in the electrode chamber. The plasma membrane of cells was selectively permeabilized using 0.03% digitonin (Sigma) in DMSO. Respiration was measured using the oxidizable substrates, 5 mM/L malate and 5 mM/L glutamate. State 3 respiration (active oxidative phosphorylation) was measured after the addition of 1.6 mM/L ADP. State 4 respiration (resting respiration limited by the inner membrane proton permeability) was measured after the addition of the ATP synthase poison oligomycin at 5 μg/mL (Sigma; ref. 30). Rates for both state 3 and 4 respiration were recorded in units of nanomol O₂/min/10⁶ cells. In two experiments, respiration by intact, nonpermeabilized cells was measured in sodium chloride buffer plus glucose (see above) but in the absence of other oxidizable substrates and ADP. Under these conditions, endogenous respiration was measured before and after the addition of oligomycin.

Cytochrome c oxidase activity assay. Cytochrome c oxidase enzyme activity was monitored by the oxidation of reduced cytochrome c at 550 nm (31). Cells were trypsinized, rinsed twice with PBS, and resuspended in 10 mM/L phosphate (KH₂PO₄/K₃HPO₄) buffer (pH 7.4) containing 20 mM/L succinate. Cells were then lysed using 0.5% Triton X-100. Cytochrome c (Sigma) was dissolved in 10 mM/L phosphate buffer in the presence of 0.1 mol/L L-ascorbate as a reductant, which was later removed using a Sephadex G-25 column. Reduced cytochrome c (20 μM/L) was added to lysates containing 30 μg protein in 1 mL of 20 mM/L phosphate buffer, and decreases in absorbance, which indicate cytochrome c oxidation, were recorded for 2 minutes. Activity was determined by assessing the rate and extent of enzymatic cytochrome c oxidation and calculated in units of nanomol cytochrome c oxidized/min/mg protein with an extinction coefficient of 19 per mmol/L/cm (32).

Western blotting for manganese SOD. Exponentially growing cells were washed twice with cold PBS and protein was isolated from cells scraped off 75-cm² flasks. Cells were then lysed with buffer containing 500 mM/L NaCl, 50 mM/L Tris (pH 8.0), 1% NP-40, and protease inhibitors (Calbiochem, San Diego, CA) and complete cell lysis was ensured by sonication. Lysates were centrifuged at 14,000 rpm at 4°C and protein concentration of the collected supernatants was determined through a Bradford protein assay (Bio-Rad). Proteins (30 μg) were electrophoresed on 4% to 12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA) in MES buffer and transferred by electrophoresis to polyvinylidene fluoride membranes (Invitrogen). Proteins on the membrane were incubated with a rabbit polyclonal anti-manganese SOD (MnSOD) primary antibody (Upstate, Charlottesville, VA). A secondary anti-rabbit antibody and chemiluminescent substrate solution (KPL, Inc., Gaithersburg, MD) was used to visualize these bands and Quantity One software was used to quantify protein band intensities in digital images of the blot. A loading control, blots were also incubated with mouse anti-glyceraldehyde-3-phosphate dehydrogenase antibodies (StressGen, Victoria, British Columbia, Canada).

MnSOD activity. MnSOD activity was measured using a spectropho- metric assay (R&D Systems, Minneapolis, MN). Cell extracts were prepared from exponentially growing cultures. Activity was determined as per the manufacturer’s instructions using a colorimetric assay based on the ability
of SOD to form H₂O₂ from superoxide radicals generated by an exogenous reaction involving xanthine and xanthine oxidase. The extent of prevention of a colorimetric reaction involving superoxide and a superoxide scavenging molecule, nitroblue tetrazolium, indicated the activity of MnSOD. Activity was measured using 50 μg protein lysate per cell clone in the presence of 5 mmol/L sodium cyanide (NaCN; Sigma) to inhibit copper/zinc SOD (CuZnSOD) activity, leaving MnSOD activity unchanged. Mitochondrial and microsomal fractions produce free radicals that reduce the sensitivity of the assay especially on addition of NaCN. Bathocuproine sulfate (BCS; 0.05 mmol/L) and fatty acid-free bovine serum albumin (BSA; 0.13 mg/mL; Sigma) were added to the reaction to prevent mitochondrial electron transport oxidant production that interfered with measurements. BCS is an iron chelator that blocks the electron transport chain effectively without interfering with this assay and the BSA increases BCS solubility (33). Absorbance changes were recorded for 5 minutes and rates were calculated for three independent sets of lysates for each of the clones.

**Statistical analysis.** Data are expressed as the mean of three independently replicated experiments ± SE unless otherwise indicated. Statistical significance was taken as \( P < 0.05 \) and determined by one-way ANOVA followed by the Dunnett’s test.

### Results

**Chromosomal instability.** Instability is characterized by chromosomal rearrangements visualized through metaphase analysis of chromosomes with the hamster chromosome by fluorescence in situ hybridization (21, 22). The two unstable clones used in this study, LS12 and Fe10-3, were derived from irradiated surviving GM10115 cells using ionizing radiation. These unstable clones contain several numbers of different subpopulations of rearranged metaphase chromosomes and were used for subsequent studies with the unirradiated GM10115 cell line.

**Total ROS levels and mitochondrial contribution to H₂O₂ production in stable and unstable clones.** Evidence of oxidative stress, including increased ROS levels and lipid peroxidation, was previously found in unstable clones generated from irradiated clonal expansions of GM10115 cells (8, 9, 12). To confirm persistent oxidative stress in our unstable clones, cells were incubated with the free radical indicator probe, DCFDA. Measuring fluorescence emission with flow cytometry, unstable cells showed higher levels of ROS than the stable parental cell line. Figure 1A shows that LS12 and Fe10-3 cells present a 36% and 70% increase in ROS levels, respectively, over the parental GM10115 cell line (\( P = 0.004 \)).

To determine relative mitochondrial contribution to the increased ROS levels in the unstable cells (i.e., the differences between the clones in mitochondrial production of electron transport–generated ROS to the total cellular ROS levels), a fluorometric Amplex Red assay was used to detect real-time rates of cellular H₂O₂ production. The mitochondrial contribution to the total cellular H₂O₂ production was defined as the difference in the rate of Amplex Red fluorescence in the absence and presence of the protonophoric uncoupler molecule, FCCP. FCCP reduces H₂O₂ production by isolated mitochondria by up to 70% (24). Although this calculation somewhat underestimates the mitochondrial contribution, unstable cells showed a greater percentage decrease in ROS after addition of FCCP than GM10115 cells. LS12 and Fe10-3 ROS levels decreased by about 43% and 46%, respectively, and the parental cell line decreased by 34% (\( P = 0.003; \) Fig. 1B). The degree to which FCCP reduces mitochondrial ROS production is dependent on the level of metabolism relative to state 3 respiration (low rate of ROS production) versus state 4 respiration (high rate). To determine the relative rates of endogenous mitochondrial metabolism in the cell lines, \( O_2 \) electrode measurements of respiration by intact cells in the absence and presence of the ATP synthetase inhibitor oligomycin were made. The ratio of rates obtained in the absence compared with the presence of oligomycin ranged from 2.0 to 2.3 (\( n = 2 \)) and was the same for each cell type, indicating that the differences in FCCP-sensitive H₂O₂ production are not due to differences in cellular metabolism. Thus, the mitochondrial contribution to cellular H₂O₂ generation is greater in the unstable
cells, likely due to some inherent alteration in the number of mitochondria or specific electron transport activities.

**Mitochondria levels.** Because increased numbers of mitochondria may be the source of the increased ROS levels in the unstable cells, we examined mitochondrial amounts in each of the clones. Because the Mitotracker Green FM probe stains mitochondria in a membrane potential-independent manner, it is a reliable indicator of mitochondrial levels (25). Flow cytometry measurements indicate that mitochondrial levels are not significantly different among the three cell lines (Fig. 2A).

Levels of mtDNA can also reflect amounts of mitochondria. MtDNA levels were quantified through Southern blotting (34, 35) for two mtDNA encoded genes, COX2 and COX3, using β-actin DNA for normalization. There were no significant differences in mtDNA levels between cell lines (Fig. 2B and C).

**Respiration.** Although respiration produces ATP for cellular processes, a harmful by-product of electron transport is the superoxide radical formed when electrons do not completely transfer through the electron transport chain and react with molecular oxygen. Increased superoxide radical production occurs with respiratory defects or blockage (36, 37). To determine whether altered respiration may be the cause of the higher ROS levels in unstable cells, we measured respiratory rates of mitochondria in whole cells with plasma membranes permeabilized by digitonin. Digitonin can be used to permeabilize cell membranes, whereas intact mitochondria respiration is measured in situ in a physiologically relevant environment (38). In contrast to intact cells where metabolic demand normally limits the rate of respiration, the rate of O2 consumption by permeabilized cells can be measured at maximum and minimum rates using high levels of ADP and oligomycin, respectively. As seen in Fig. 3A, the state 3 respiration rates for both unstable lines were ~40% lower than the parent cell line (GM10115: 106.8 nmol O2/min/107 cells; LS12, 62.6 nmol O2/min/107 cells; Fe10-3, 60.3 nmol O2/min/107 cells; P < 0.01), whereas rates of state 4 respiration were not significantly different. These results suggest all or a portion of the chain may be altered in a way that ultimately inhibits oxygen uptake. One set of additional experiments was done to verify that the electron transport chain rather than the ATP synthase or adenine nucleotide translocase is responsible for the decrease in state 3 respiration observed in the unstable cell lines. Following the addition of oligomycin, the protonophore uncoupler FCCP (10 μmol/L) was added and the rate of uncoupled respiration was measured, thereby bypassing the activity of enzymes involved in ATP synthesis and transport. Compared with the GM10115 cells, uncoupled respiration in LS12 and Fe10-3 cells was reduced by 42% and 27%, respectively. This degree of reduction was similar to the impairment observed in state 3 respiration (50% and 32%, respectively), thereby indicating that the electron transport chain or possibly tricarboxylic acid cycle enzymes are responsible for the reduced state 3 respiration observed in unstable cell lines.

Therefore, we examined complex IV, cytochrome c oxidase, which is the final step in the respiratory chain where oxygen is reduced to a water molecule while cytochrome c becomes oxidized. We determined complex IV function by measuring the rate of oxidation of exogenous reduced cytochrome c using a spectrophotometric complex IV activity assay. Figure 3B shows the results of complex IV activity; LS12 and Fe10-3 show a 28% and 32% lower activity, respectively, compared with GM10115 (P < 0.001).

**MnSOD protein level and enzyme activity determination.** The oxidant status in our cell lines is dependent on both oxidant processes, a harmful by-product of electron transport is the superoxide radical formed when electrons do not completely transfer through the electron transport chain and react with molecular oxygen. Increased superoxide radical production occurs with respiratory defects or blockage (36, 37). To determine whether altered respiration may be the cause of the higher ROS levels in unstable cells, we measured respiratory rates of mitochondria in whole cells with plasma membranes permeabilized by digitonin. Digitonin can be used to permeabilize cell membranes, whereas intact mitochondria respiration is measured in situ in a physiologically relevant environment (38). In contrast to intact cells where metabolic demand normally limits the rate of respiration, the rate of O2 consumption by permeabilized cells can be measured at maximum and minimum rates using high levels of ADP and oligomycin, respectively. As seen in Fig. 3A, the state 3 respiration rates for both unstable lines were ~40% lower than the parent cell line (GM10115: 106.8 nmol O2/min/107 cells; LS12, 62.6 nmol O2/min/107 cells; Fe10-3, 60.3 nmol O2/min/107 cells; P < 0.01), whereas rates of state 4 respiration were not significantly different. These results suggest all or a portion of the chain may be altered in a way that ultimately inhibits oxygen uptake. One set of additional experiments was done to verify that the electron transport chain rather than the ATP synthase or adenine nucleotide translocase is responsible for the decrease in state 3 respiration observed in the unstable cell lines. Following the addition of oligomycin, the protonophore uncoupler FCCP (10 μmol/L) was added and the rate of uncoupled respiration was measured, thereby bypassing the activity of enzymes involved in ATP synthesis and transport. Compared with the GM10115 cells, uncoupled respiration in LS12 and Fe10-3 cells was reduced by 42% and 27%, respectively. This degree of reduction was similar to the impairment observed in state 3 respiration (50% and 32%, respectively), thereby indicating that the electron transport chain or possibly tricarboxylic acid cycle enzymes are responsible for the reduced state 3 respiration observed in unstable cell lines.

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**MnSOD protein level and enzyme activity determination.** The oxidant status in our cell lines is dependent on both oxidant...
production and antioxidant activity. Consequently, we examined MnSOD enzyme activity and immunoreactivity in our clones. We found no significant differences in MnSOD levels, and although there seems to be increased relative levels for Fe10-3, the difference is not statistically significant (Fig. 4A and B). In contrast, the MnSOD enzyme activity in the unstable cells is significantly lower (40-60%) than in the stable parental cell line. (P < 0.001; Fig. 4C).

Figure 3. Cellular respiration and cytochrome oxidase activities. A, mitochondrial respiration in situ was measured using a thermostatically controlled Clark oxygen electrode. Cells (1 x 10^7) were resuspended in 0.5 mL sodium buffer (140 mmol/L NaCl, 3 mmol/L KCl, 0.4 mmol/L KH_2PO_4, 20 mmol/L HEPES, 5 mmol/L NaHCO_3, 5 mmol/L glucose, 1 mmol/L MgCl_2) at 37°C. The plasma membrane was selectively permeabilized with 0.03% digitonin in DMEM. Malate (5 mmol/L) and glutamate (5 mmol/L) and ADP (1.6 mmol/L) were added to initiate state 3 respiration. Oligomycin (5 µg/mL), an ATPase synthase inhibitor, was added to initiate state 4 respiration. Columns, mean respiratory rates for state 3 and 4 respiration (n = 4); bars, SE. P < 0.01. B, cytochrome oxidase activity was measured using cells lysed with 0.5% Triton X-100 in 10 mmol/L phosphate (KH_2PO_4/K_2HPO_4) buffer containing 20 mmol/L succinate (pH 7.4) at room temperature. Reduced cytochrome c was added to cell lysates containing 30 µg protein in 20 mmol/L phosphate buffer. The rate of cytochrome c oxidation was determined over 2 minutes using a spectrophotometric assay at 550 nm. Values represent activities of unstable cells as a percentage of activity of stable cells. (n = 10; P < 0.001).

Figure 4. Manganese superoxide immunoreactivity and enzyme activity. A, total cellular protein was isolated and Western blotting was done for MnSOD for all three clones. β-Actin was used to normalize values. B, densitometric quantification of MnSOD immunoreactivity present in cell lysates was normalized for each Western immunoblot to β-actin levels (n = 3). C, MnSOD enzyme activity present in 50 µg cell lysate protein was measured by the ability of enzyme to prevent a colorimetric reaction involving nitroblue tetrazolium and superoxide anions generated by an exogenous reaction involving xanthine oxidase. NaCN (5 mmol/L) was added to inhibit CuZnSOD leaving MnSOD activity unaffected. BSA (0.13 mg/mL) and BCS (0.05 mmol/L) were added to eliminate electron transport–generated oxidants. For three independent experiments with three sets of lysates, absorbance changes were measured over 5 minutes at 550 nm. Units of activity were determined through a standard curve. (P < 0.001).
Discussion

Mitochondria-induced oxidative stress is implicated in many diseases, including cancer (18, 39). Morphologic changes in the mitochondria have also been seen in association with cancer (40) and in response to oxidative stress caused by respiratory defects and cytochrome oxidase inhibition (41). In a study of nontransformed NIH3T3 cells, most ROS production derives from cytosolic NADPH oxidase, whereas, in transformed liver and colon tumor cells, the increased ROS production is of mitochondrial origin (42). Some suggested mechanisms by which dysfunctional mitochondria may lead to carcinogenesis are apoptotic resistance or increased ROS leading to either (a) direct damage to crucial homeostatic genes or (b) activation of tumorigenic signaling pathways [e.g., those involving nuclear factor-κB (NF-κB) or activator protein (AP-1); ref. 43]. As genomic instability may play a role in carcinogenesis, understanding the initiation and perpetuation of RIGI may elucidate the mechanisms of tumorigenesis (44).

In investigating the role of mitochondria in RIGI, we found function differences between the mitochondria of stable and unstable clones. Mitochondria within genetically unstable cells contribute more to total cellular ROS production than those of stable cells (Fig. 2B). This mechanism of ROS production may be responsible for the elevated cellular superoxide levels, measured by dichlorofluorescein fluorescence, and elevated lipid oxidation documented previously with the same genomically unstable cell lines used in this study (7). The elevated respiratory uncoupler-sensitive H_{2}O_{2} production is not due to an abnormally high level of mitochondria (Fig. 4) or to differences in cellular energy metabolism. One possible explanation for increased mitochondrial ROS production is respiratory inhibition (e.g., caused by gene mutations or impaired gene expression; ref. 16). Oxidative stress due to increased mitochondrial ROS formation in response to respiratory defects (36, 37) may in fact contribute to tumorigenesis (45).

Although electron transport chain complexes I and III are the primary sites of mitochondrial ROS production (46), inhibition of complex IV (cytochrome oxidase) can induce the formation of ROS at these proximal sites of electron flow (47). Moreover, cyanide inhibition of complex IV induces an oxidative stress–mediated cell transformation (48). We therefore compared complex IV enzyme activity in the unstable cells and observed a significantly reduced cytochrome oxidase redox activity in the unstable cells (Fig. 3F), which correlates with their decreased state 3 respiratory activity (Fig. 3A). The cause of reduced cytochrome oxidase activity is, at this juncture, unknown. Additional measures of electron transport chain complex activities are in progress to determine if impairment is specific or generalized. Despite the reduced cytochrome oxidase activity and state 3 respiration measured in digitonin-permeabilized genomically unstable cells, rates of respiration by intact stable and unstable cells are very similar. Although the respiratory defects do not seem to limit basal cellular aerobic energy metabolism, the increased DCF fluorescence and elevated mitochondrial contribution to cellular H_{2}O_{2} production strongly suggest that the respiratory dysfunction contributes to elevated oxidative stress in the unstable cell lines.

Another possible contributor to oxidative stress in the unstable clones is reduced MnSOD activity. Although the unstable clones exhibit reduced enzymatic activity (Fig. 4B), immunoreactive levels of the MnSOD protein are similar (Fig. 4A). The decreased enzymatic activity could be due to increased ROS as MnSOD is subject to inactivation by several reactive oxygen and nitrogen species, including H_{2}O_{2} (49, 50). Although reduced MnSOD activity could contribute to the increased DCF fluorescence seen in the unstable cells, it does not explain elevated mitochondrial contribution to cellular H_{2}O_{2} production. The Ampex Red method for measuring H_{2}O_{2} production precludes the detection of altered MnSOD activity as exogenous CuZnSOD is included in the assay medium primarily to reduce background Ampex Red oxidation. In addition, the cellular CuZnSOD activity and spontaneous superoxide dismutation likely mask any defect in MnSOD activity when measuring cellular H_{2}O_{2} production. A cycle of perpetuated oxidative stress may be occurring in the unstable cell system that is promoted by decreased MnSOD activity, mitochondrial molecular alterations, respiratory inhibition, and elevated mitochondrial ROS production. Decreased MnSOD activity is found in most cancers (51), whereas up-regulation of MnSOD suppresses tumor growth (52). Evidence also suggests that MnSOD helps maintain genomic stability. Heterozygous MnSOD knockout fibroblasts experience a loss of cell viability, increased mitochondrial oxidative stress, and increased chromosomal abnormalities, including double-strand breaks, end-to-end fusions, and chromosomal translocations (53).

We have shown mitochondrial abnormalities in the unstable cells and that they contribute to the oxidative stress seen in RIGI. These results extend initial studies by Nagar et al. (54) where unstable clones showed increased cytochrome c release and variations in apoptotic protein profiles. What we suspect may be occurring is a combination of events that each lead to overall cellular oxidative stress, including mitochondrial dysfunction, stress signaling pathways, including NF-κB (55) or AP-1, or cytokine signaling pathways, involving transforming growth factor-β or interleukin-8, which increase ROS levels. We are currently testing these possibilities. How ROS is involved in genetic instability is unclear. In addition to direct ROS-mediated cellular damage, global processes involved in genome stabilization are probably disrupted. Our results contribute to the growing evidence that mitochondrial abnormalities are involved in oncogenesis through its role in nuclear genome destabilization.

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Grace J. Kim, Gary M. Fiskum and William F. Morgan


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