Persistent Oxidative Stress in Chromosomally Unstable Cells

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ABSTRACT

Past work using the human-hamster hybrid line GM10115 has demonstrated that exposure to a variety of DNA damaging agents can lead to the persistent destabilization of chromosomes. To gain insight into the potential biochemical mechanisms involved in perpetuating the unstable phenotype, groups of clones characterized as stable or unstable were analyzed for indications of oxidative stress. All of the clones were derived from single progenitor cells surviving exposure to ionizing radiation or chemicals. Compared with their stable counterparts, unstable clones possessed elevated levels of reactive oxygen species (ROS) as measured by their enhanced ability to oxidize fluorogenic dyes. Fluorescence automated cell sorting analysis indicated that unstable clones had significantly higher mean fluorescence signals of ~2-fold and ~1.25-fold, respectively, as derived from the dyes 5-(and-6)-chloromethyl-2,7'-dichlorodihydrofluorescein diacetate and dihydrorhodamine 123, respectively. To determine whether mitochondria might constitute a potential source of ROS, stable and unstable clones of cells were analyzed for mitochondrial content using nonyl acridine orange and function using rhodamine 123. Fluorescence automated cell sorting data indicated that compared with stable clones, unstable clones possessed an elevated number (15% increase in mean nonyl acridine orange fluorescence) of dysfunctional mitochondria (27% decrease in mean rhodamine 123 fluorescence). Interestingly, the consequences of elevated ROS did not translate to an increase in oxidative base damage in nuclear DNA. Analysis of nine different base damage adducts by gas chromatography/mass spectrometry did not reveal significant differences between stable and unstable clones. The data suggest that the perpetuation of many of the abnormal phenotypes associated with genomic instability may be linked to a state of chronic oxidative stress derived in part from dysfunctional mitochondria.

INTRODUCTION

Exposure of cells to a variety of genotoxic and cytotoxic agents have the potential to elicit prolonged and dynamic changes that compromise the stability of the cellular genome (1). Many of these changes, whether induced directly or indirectly by DNA damage, lead to increases in gene mutation and amplification, reduced cloning efficiency, elevated micronuclei, sister chromatid exchanges, and multiple karyotypic abnormalities (1). These endpoints characteristic of genomic instability provide evidence for a prolonged disruption to cellular homeostasis that perpetuates the memory of past insult over multiple cellular generations. In efforts to understand the biochemical basis of persistent genomic flux, past work has studied the types of agents that lead to genomic instability (2), the relationship between multiple endpoints of genomic instability (3), and the consequences of developing an unstable phenotype (4). Whereas we have shown that DNA damage is a critical factor contributing to the eventual manifestation of chromosomal instability (2, 5), others have demonstrated the involvement of other important factors. Evidence in support of the latter comes from studies designed to determine whether non-nuclear damage is sufficient to elicit characteristic endpoints of genomic instability. The use of single-cell microirradiators that can target damage to the cytoplasm has been found to increase mutations and oxidative base damage (6). Furthermore, studies estimating the number of cells traversed by incident α-particles by Poisson statistics (7–10), and more recently refined through the experimental manipulation of cellular shielding protocols (11), provide evidence that genomic instability can be induced in cells that did not incur direct DNA damage. A wealth of related data has demonstrated that populations of undamaged cells can exhibit radiomimetic changes when coincubated with cells or conditioned medium derived from irradiated cultures (12–17). These “bystander effects” support the idea that damage-inducible end points can be transmitted between populations of damaged and undamaged cells. Thus, whereas cells that incur genotoxic damage are more likely to develop downstream abnormalities, a satisfactory explanation for the development of genomic instability must incorporate the role of non-nuclear targets within the context of the extracellular environment.

There is evidence for increased levels of ROS in genomically unstable clones produced by exposure to ionizing radiation (4, 18). The persistence of ROS indicates a continual turnover of oxidative species, and suggests that conditions of chronic versus acute oxidative stress may contribute to the development and/or maintenance of genomic instability. Data demonstrating the induction of genomic instability after chronic but not acute hydrogen peroxide treatment also support this idea (19). To determine the potential significance of an altered redox state on genomic instability, we have analyzed subsets of chromosomally stable and unstable clones for indications of oxidative stress. These studies indicate that chromosomal instability is associated with a state of oxidative stress and has prompted investigations into the possible source of the elevated ROS. As the site of electron transport, mitochondria constitute a logical source for the elevated ROS (20–22). Mitochondrial dysfunction can lead to an elevation of ROS by increasing the residence time of electrons at specific steps in the electron transport process, thereby increasing the probability of electron transfer to oxygen. Thus, we have investigated the role of the mitochondria in stable and unstable clones, to determine whether chromosomal instability is associated with altered mitochondrial levels and/or functionality. Our data suggest that oxidative stress derived from dysfunctional mitochondria may contribute to the perpetuation of the unstable phenotype and implicates oxidative stress.
as a biochemical mechanism capable of perturbing the maintenance of genomic integrity.

MATERIALS AND METHODS

Cell Lines. Isogenic groups of chromosomally unstable and stable clones were derived from the GM10115 human-hamster hybrid line. GM10115 cells contain a single copy of human chromosome 4 in a background of 22–24 hamster chromosomes, and the human chromosome is used to monitor chromosomal instability after exposure to DNA damaging agents (2). GM10115 cells were exposed to sparsely ionizing radiation (250 kVp X-rays), densely ionizing radiation (1 GeV Fe156 ions), or bleomycin (100 µg/ml, 1 h) plated and clonally expanded for analysis of potential chromosomal instability.

Chromosomal Instability. Colonies derived from single progenitor cells surviving DNA damaging treatments were picked at random and expanded to mass population for the analysis of chromosomal instability. This was based on fluorescence in situ hybridization using a probe derived from a whole human chromosome 4 specific library as described previously (2, 23). Metaphase preparations were analyzed by fluorescence microscopy for rearrangements involving the human chromosome and analysis was based on at least 200 preparations were analyzed by fluorescence microscopy for rearrangements involving the human chromosome and analysis was based on at least 200 metaphase spreads per clone. More than 1,000 clones (200,000 metaphases) were screened and selected for the present studies based on the following criterion.

Stable clones selected were those that exhibited either no chromosomal rearrangements or one aberration common throughout all of the metaphases scored. The presence of one common aberration throughout the cellular population is considered a signature of chromosome damage induced directly by the incident radiation and, therefore, does not represent chromosomal instability (3). Unstable clones selected were those that exhibited ≥10 distinct aberration types that represented a majority (>90%) of the total metaphases scored.

Reactive Oxygen Intermediates. The presence of intracellular reactive oxygen intermediates was based on the ability of cells to oxidize fluorogenic dyes to their corresponding fluorescent analogues. Exponentially growing cultures of stable and unstable clones were harvested just before assay, resuspended at 1 × 10⁶ cells/ml in tissue culture medium and incubated for 1 h with 5 µM H₂R123 (Molecular Probes, Eugene, OR) or CM-H₂DCFDA (Molecular Probes). After dye incubation cells were immediately subjected to FACS analysis, in which a minimum of 30,000 cells were sampled per condition. Intracellular oxidation of H₂R123 and CM-H₂DCFDA yields R123 and dichlorofluorescein that can be detected by FACS.

Mitochondrial Content and Function. To determine the relative quantity of mitochondria, cells were incubated with NAO (Molecular Probes) that provides a fluorescent signal proportional to mitochondrial content (24, 25). Functionality of the mitochondrial organelle was probed using R123 (Molecular Probes). Intracellular oxidation of H₂R123 and CM-H₂DCFDA yields R123 and dichlorofluorescein that can be detected by FACS.

Oxidative Base Damage. The presence of oxidative base damage was measured in DNA derived from chromosomally stable and unstable clones by a Hewlett-Packard 5890A gas chromatograph interfaced to a Hewlett-Packard 5970B mass selective detector (GC/MS; Ref. 33). DNA (50–100 µg) was isolated and extracted from cells under antioxidant conditions (0.1% butylated hydroxytoluene in the organic phase and 1 mM DTT in all of the aqueous phases), concentrated, and frozen in Tris buffer until sample preparation for GC/MS. Isolated DNA samples were acid hydrolyzed and trimethylsilylated for analysis by GC/MS (33, 34). 8-Azaadenine (3 mM) was added to each sample, and 0.5–1.0 mM of base damage products isotopically enriched in 13C and 15N, were included as internal standards. The acid hydrolysates were lyophilized, then trimethylsilylated with 80 µl N,O-bis(trimethylsilyl)trifluoracetamide, 30 µl acetonitrile, and 5 µl of pyridine at 65°C for 4 h. The N,O-bis(trimethylsilyl)trifluoracetamide, acetonitrile and pyridine were degassed before use, and the trimethylsilylation reaction was conducted under ACS-grade nitrogen gas (oxygen content < 1 ppm) to prevent the artifactual oxidation of the normal bases in the sample (35, 36). The trimethylsilylated acid hydrolysates were then analyzed by GC/MS.

The base damage products were quantitated using either isotope dilution methods or, when isotopically enriched authentic compounds were not available, relative molar response factors based on 8-azaadenine as the internal standard, as are described elsewhere (33, 34, 37). The mean relative SDs among three irradiated clone samples and their respective duplicates ranged between 0.58 and 21 for the 10 base damage products, with 5-OHU, 5-HMU, 5-OHC, TG, FpyA, FpyG, and 8oxoG ranging between 0.58 and 7.7.

Statistics. Significance between data sets obtained through FACS analysis was determined by the K-S test provided with the Cell Quest software. This two-sample test returns a P based on the differences between data sets. Fluorescent values derived from FACS data are presented as RFUs. Other data were averaged, and the means of all of the stable and unstable clones were analyzed by ANOVA and t tests.

RESULTS

Chromosomally Stable and Unstable Clones. The cell system used for this study has been described in past reports (2, 23) and contains a single human chromosome 4 that is used to monitor chromosomal rearrangements involving this chromosome by fluorescence in situ hybridization. Randomly selected clones surviving various DNA damaging treatments were expanded to mass population and analyzed for chromosomal instability. Clones showing a minimum of 10 aberrant subpopulations comprising at least 90% of the total metaphase population scored were considered unstable, whereas stable clones were selected from those clones that exhibited no more than two aberrant subpopulations (Table 1).

Intracellular Levels of Reactive Oxygen Intermediates. To determine whether chromosomal instability was associated with persistent oxidative stress, stable and unstable clones treated with CM-H₂DCFDA or H₂R123 were analyzed by FACS. The ability of cells to oxidize these dyes provides a measure of the intracellular level of ROS (38–40). FACS histograms shown in Fig. 1 represent the average of all of the clones analyzed. Comparison of the mean CM-H₂R123, NAO and R123 fluorescence (Table 1).

| Table 1 Cytogenetic characteristics of chromosomally stable and unstable clones |
|------------------------------------|-------------------|-------------------|--------------|
| Stable clones                      | DNA damaging agent| Dose/concentration| No. of metaphase subpopulationsa | % of aberrant metaphasesb |
| S1                                 | X-rays            | 10 Gy             | 0            | 0            |
| S2                                 | X-rays            | 10 Gy             | 0            | 0            |
| S3                                 | X-rays            | 10 Gy             | 0            | 0            |
| S4                                 | X-rays            | 10 Gy             | 0            | 0            |
| S5                                 | X-rays            | 10 Gy             | 0            | 0            |
| S6                                 | X-rays            | 10 Gy             | 0            | 0            |
| S7                                 | X-rays            | 10 Gy             | 1            | 3.5           |
| S8                                 | X-rays            | 10 Gy             | 2            | 2            |
| S9                                 | X-rays            | 10 Gy             | 1            | 93           |
| S10                                | X-rays            | 10 Gy             | 1            | 100          |

Unstable clones

| U1                                 | X-rays            | 7.5 Gy            | 14           | 95          |
| U2                                 | X-rays            | 7.5 Gy            | 14           | 95          |
| U3                                 | X-rays            | 10 Gy             | 100          |
| U4                                 | X-rays            | 10 Gy             | 100          |
| U5                                 | X-rays            | 10 Gy             | 21           |
| U6                                 | X-rays            | 10 Gy             | 12           |
| U7                                 | X-rays            | 15 Gy             | 14           |
| U8                                 | Fe56 ions         | 7.5 Gy            | 13           |
| U9                                 | Bleomycin         | 100 µg/ml         | 11           |
| U10                                | Bleomycin         | 100 µg/ml         | 16           |

a Number of different abnormal metaphase subpopulations showing distinct types of chromosomal rearrangements involving human chromosome 4.

b Percentage of metaphases scored showing chromosomal aberrations.

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H$_2$DCFDA fluorescence of stable (4.66 RFU) and unstable (9.60 RFU) clones indicates that unstable clones show a statistically significant increase in fluorescence of 106% ($P < 0.01$ by the K-S test). Qualitatively similar data were obtained when the same groups of clones were analyzed using H$_2$R123 (Fig. 2). Unstable clones demonstrated an enhanced ability to oxidize H$_2$R123, as indicated by a significant 24% increase in mean fluorescence (22.2 RFU) compared with the mean fluorescence found in stable clones (17.9 RFU; $P < 0.01$ by the K-S test). Data obtained from both dyes reveal qualitatively similar results and suggest that populations of unstable clones possess higher intracellular levels of ROS than their chromosomally stable counterparts.

Mitochondria as Potential Sources of Elevated Reactive Oxygen Intermediates. To determine whether dysfunctional mitochondria in unstable clones were a source of elevated ROS, cells were analyzed with two dyes that are specific and sensitive to changes in mitochondrial function (R123) and content (NAO). As a group, the unstable clones exhibited a reduced signal for R123 compared with stable clones (Fig. 3). The mean fluorescence of R123 in unstable clones was 27% lower relative to the stable clones, a difference found to be statistically significant (K-S test, $P < 0.01$). Because R123 fluorescence is dependent on the mitochondrial content probe NAO (10 RFU) or mitochondrial function probe R123 (10 RFU) for 30 min before FACS analysis, histograms shown correspond to the average of all stable (---) or unstable (-----) clones analyzed in two separate experiments. Compared with stable clones, the histograms shown for unstable clones reveal a relative increase and decrease in NAO and R123 fluorescence, respectively, indicating an increase in the number of dysfunctional mitochondria. FACS analysis of undamaged controls (GM10115 parental line) lie within the histograms shown for the stable clones (data not shown).

Fig. 3. Chromosomally unstable clones possess an increased level of dysfunctional mitochondria. Chromosomally stable and unstable clones were incubated in the presence of the mitochondrial content probe NAO (10 µM) or mitochondrial function probe R123 (10 µM) for 30 min before FACS analysis. Histograms shown correspond to the average of all stable (---) or unstable (-----) clones analyzed in two separate experiments. Compared with stable clones, the histograms shown for unstable clones reveal a relative increase and decrease in NAO and R123 fluorescence, respectively, indicating an increase in the number of dysfunctional mitochondria. FACS analysis of undamaged controls (GM10115 parental line) lie within the histograms shown for the stable clones (data not shown).

To determine whether the relative shift in R123 fluorescence between stable and unstable clones might be because of differences in the number of mitochondria, clones were analyzed for NAO fluorescence. Contrary to the results obtained with R123, the mean NAO fluorescence was 15% higher in unstable versus stable clones, a statistically significant difference (K-S test, $P < 0.01$). NAO fluorescence depends on mitochondrial content, as opposed to function, suggesting that unstable clones possess higher numbers of these organelles (Fig. 3).

Consequences of ROS. The FACS data provide evidence that unstable clones show persistent oxidative stress, so to determine whether this could elicit genetic damage, stable and unstable clones were analyzed by GC/MS for oxidative base damage. Analysis of nine distinct base adducts was determined, and the average adduct yields in undamaged controls (GM10115 cells, dark bars), stable (light bars), and unstable (intermediate bars) clones is shown in Fig. 4. The data indicate that whereas differences exist in the yield of specific base adducts (e.g., FpyG), there is no discernable trend that indicates these yields are significantly different between the stable and unstable clones.

DISCUSSION

To elucidate potential biochemical mechanisms that might contribute to the perpetuation of genomic instability, we have analyzed subsets of chromosomally stable and unstable subclones for indications of oxidative stress. Subclones derived from single progenitor cells surviving genotoxic insult were analyzed cyto-genetically to determine the status of chromosomal stability. Determination of chromosomal instability was based on both the quality (number of unique subpopulations) and quantity (number of aberrant metaphases) of chromosomal rearrangements involving the marker human chromosome 4, and was the only criterion used to distinguish each group of subclones. Subsequent analysis of all of the subclones selected in this
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Fig. 4. Oxidative base damage in chromosomally stable and unstable clones. Exponentially growing cultures of all clones were harvested for the isolation and purification of genomic DNA in the presence of antioxidants. DNA samples were then degraded to individual bases for analysis by GC-MS as described (see “Materials and Methods”). Bar charts show the average level of base damage detected in all stable (light shading) and unstable (intermediate shading) clones for each of the nine adducts analyzed: 8-oxoG, 2-oxoA, adenine; 8-oxoA, 5-hydroxycytosine; TG, thymine glycol; FpyG, Fpy-adenine; 8-oxoA, 8-oxo-7,8-dihydroadenine; 2-oxoA, 2-oxo-7,3-dihydroadenine; FpyG, Fpy-guanine; 8-oxoG, 8-oxo-7,8-dihydropyrimidine.

manner occurred some 25–35 cell doublings after exposure to the various DNA damaging agents used to elicit the unstable phenotype.

To determine whether evidence of a persistent oxidative stress could be found associated with the unstable phenotype, we focused on the use of fluorogenic dye precursors. FACS analysis of the fluorescent products derived from the intracellular oxidation of the parent compounds reveals that as a group chromosomally unstable clones possess elevated levels of ROS. Analysis of stable (S1–10) and unstable (U1–10) clones incubated with two different fluorogenic precursor dyes (CM-H2DCFDA and H2-R123) yields qualitatively similar data; FACS histograms averaged from all of the individual clones show that each dye is oxidized more extensively in unstable clones. Because data were averaged from groups of isogenic subclones, it is unlikely that the higher fluorescence observed in unstable clones could be accounted for by variations in the uptake and/or retention of the fluorogenic dyes between stable and unstable clones. Similarly, individual outliers cannot account for the shift in the FACS histograms shown in Figs. 1 and 2. Whereas the ROS-sensitive dyes exhibit similar trends in stable and unstable clones, each of these chemically distinct compounds can be expected to possess different oxidation potentials and to exhibit different intracellular distributions, factors that make direct comparisons of fluorescent yields between each dye difficult. Nonetheless, the data suggest that chromosomal instability is associated with a state of elevated oxidative stress.

Data finding that unstable clones possessed elevated levels of ROS corroborated our past results (4) demonstrating similar findings and prompted additional studies aimed at identifying the potential source of the elevated ROS. Our past work showing a higher incidence of apoptosis in chromosomally unstable clones suggested that the degradation of apoptotic cells might contribute to an excess of ROS (4). However, given the limited diffusion distances of ROS (41), it is unlikely that ROS derived from extracellular sources can account for the majority of the intracellular oxidation of fluorogenic dyes. Consequently, we sought to determine whether dysfunctional mitochondria might contribute to the intracellular pool of ROS.

Mitochondrial content and function were probed using the cell-permeable dyes NAO and R123, respectively. NAO fluorescence provides a relative measure of the net mitochondrial mass, whereas R123 fluorescence is sensitive to the transmembrane potential and provides a relative measure of mitochondrial function (28, 29). FACS histograms derived from the average of all of the clones indicated that chromosomally unstable clones possessed an elevated number of dysfunctional mitochondria. The sensitivity and specificity of the R123 signal was confirmed through the use of the protonophore FCCP. This poison is a potent uncoupler of oxidative phosphorylation (32) and disrupts the transmembrane potential leading to a relative decrease (~42%) in R123 fluorescence. The dependence of R123 fluorescence on oxidative phosphorylation suggests that perturbations to mitochondrial function involve disruption to the electron transport chain. Interruptions to the flow of electrons can lead to bottlenecks that increase the residence time of electrons at certain protein complexes along the transport chain (42). This, in turn, can increase the probability that electrons will react with oxygen and lead to increases in the intracellular pool of ROS. Given the foregoing considerations, data suggest that the elevated ROS found in unstable clones may be derived from an increased number of dysfunctional mitochondria.

Whereas the mechanism underlying the increase in mitochondrial mass is not known, decreased mitochondrial function may trigger a compensatory response in cells that induces mitochondrial biogenesis. The drop in overall cellular mitochondrial function may be because of a subpopulation of mitochondria that exhibit complete or partial dysfunction. Whereas some of the mitochondrial differences between stable and unstable cells might be explained by an altered cell cycle distribution, the similarity of cell cycle profiles derived from stable and unstable clones analyzed in the past argue against this possibility. Interestingly, the induction of apoptosis and differentiation in colon carcinoma cells by herbimycin A treatment led to similar increases in dysfunctional mitochondria (43). Present and past data (4) suggest that pathways regulating mitochondrial proliferation and homeostasis are disrupted in certain cell types exhibiting indications of apoptosis and genomic instability.

To determine whether elevated levels of intracellular ROS detected in unstable clones would lead to corresponding increases in nuclear oxidative base damage, DNA extracted and purified from all of the clones was analyzed for nine different base adducts by GC/MS. Despite differences in the net yields of certain base adducts (FpyG), unstable clones as a group did not exhibit a statistically significant trend toward increased base damage. It is unlikely that the inability to detect increased base damage in unstable clones is because of the sensitivity of the GC/MS technique. Power calculations (>90% probability of rejecting the H0: μH = μU when the hypothesized mean difference in the means of the unstable clones is 24% more than the means of the stable clones) indicate that the GC/MS methodology would be capable of detecting the potential increase in base damage that would correspond to the minimum increase in ROS detected using the fluorogenic dye H2-R123 (i.e., 24%). The efficient removal of endogenous base damage by base excision repair pathways also suggests that the elevated ROS detected in unstable clones may be insufficient to increase the net burden of base damage in these cells (44). Although ROS may explain the increased mutagenesis and base damage observed after cytoplasmic irradiation (6), present results did not find a correlation between the two. Whereas differences between the two experimental systems may underlie this apparent discrepancy, our recent work exploiting the Comet Assay reveals no difference in strand break yields between stable and unstable clones (45). This strongly supports the contention that elevated ROS detected in the unstable clones does not lead to an increase in genomic oxidative base damage in these cells.

The present studies have used subsets of clones characterized cytogenetically to be either chromosomally stable or unstable. Side by side analyses of these clones has determined that chromosomal insta-
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bility is associated with a state of elevated oxidative stress. As a group, unstable clones exhibited increased ROS and an elevated number of dysfunctional mitochondria compared with stable clones. However, these differences did not translate to meaningful trends when comparing base damage yields between each group of clones. Nonetheless, the correlation between genomic instability and oxidative stress corroborates past data (4), and suggest a potential mechanistic link between chromosomal instability and the production of mitochondrially derived ROS. Recent data demonstrating elevated ROS and chromosomal breakage in DSBR repair-deficient cells corroborates the possibility that changes in oxygen metabolism affect genomic stability (46). Related data showing that oncogene-induced genomic instability involves elevated ROS (47) also adds to our present findings, and provides additional evidence that oxidative stress may underlie a number of the abnormalities associated with genomic instability and cancer progression.

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