Induction of Chromosomal Instability by Chronic Oxidative Stress[1]

Charles L. Limoli and Erich Giedzinski

Department of Radiation Oncology, University of California, San Francisco, CA 94103-0806, USA

Abstract

Earlier studies using GM10115 cells analyzed the capability of different DNA-damaging agents to induce genomic instability and found that acute oxidative stress was relatively inefficient at eliciting a persistent destabilization of chromosomes. To determine whether this situation would change under chronic exposure conditions, the human–hamster hybrid line GM10115 was cultured under conditions of oxidative stress. Chronic treatments consisted of 1-hour incubations using a range of hydrogen peroxide (25–200 μM) or glucose oxidase (GO; 5–50 mU/ml) concentrations that were administered once daily over 10 to 30 consecutive days. The toxicity of chronic treatments was modest (~one log kill) and consistent with the low yield of first division aberrations (<5%). However, analysis of over 180 clones and 36,000 metaphases indicated that chronic oxidative stress led to a high incidence of chromosomal instability. Treatment of cells with 100 and 200 μM hydrogen peroxide or 50 mU/ml GO was found to elicit chromosomal instability in 11%, 22%, and 19% of the clones analyzed, respectively. In contrast, control clones isolated after mock treatment did not show signs of chromosomal destabilization. These data suggest that chronic oxidative stress constitutes a biochemical mechanism capable of disrupting the genomic integrity of cells.
Neoplasia (2003) **5,** 339–346

Keywords: chronic oxidative stress; chromosomal instability; genomic instability; reactive oxygen species.

Introduction

The abnormal phenotypes associated with neoplastic transformation can, in many instances, be traced to an accumulation of genetic changes [1,2]. 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 including chromosomal instability [3]. The acquisition of these genetic alterations is believed to involve genomic instability, a process encompassing a wide variety of biological pathways and endpoints [3–5]. Genomic instability can be induced by a variety of damaging agents, but with varying efficiency. Agents shown to produce certain types of "complex" DNA damage containing double-strand breaks (DSBs) (e.g., ionizing radiation, bleomycin, and neocarzinostatin) are relatively efficient at inducing chromosomal instability [6]. Other agents producing a predominance of singly damaged sites (superoxide anions and hydrogen peroxide) are less efficient at inducing chromosomal instability [6,7]. These data suggest that acute oxidative stress is not an effective means to elicit genomic instability.

Reactive oxygen species (ROS), including superoxide anions and hydrogen peroxide, are metabolic byproducts produced primarily by mitochondrial respiration [8]. Superoxide is generated when electrons moving along the electron transport chain are donated directly to molecular oxygen [9–11]. Cellular defenses against these oxidizing species include various isoforms of superoxide dismutase (SOD), which convert superoxide to hydrogen peroxide, and catalase and glutathione peroxidase, which regenerate water and molecular oxygen [12]. The efficiency of these antioxidant safeguards may underlie the inefficiency with which acute oxidative stress leads to genomic instability in cells. Genetic damage arising from oxidative processes also leads to base damage, but these lesions are efficiently removed by base excision repair systems that likely promote cellular resistance to genomic instability induced by oxidative processes [13].

Although acute treatments with oxidizing agents are ineffective at eliciting persistent genomic changes, chronic oxidative stress presents cells a more difficult problem to cope with. The continual exposure of cells to pro-oxidant conditions can lead to alterations in gene expression and compensatory adaptations that can alter normal physiology. Prolonged (6-month) exposure of cells to hydrogen peroxide has been reported to elicit genomic instability [14], and there is evidence for increased levels of ROS [7,15] and lipid peroxidation endproducts [7] in genomically unstable clones produced by exposure to ionizing radiation. Addition of antioxidants has been found to reduce the frequency of chromosomal instability

Abbreviations: DSB, double-strand break; FA, Fanconi anemia; GO, glucose oxidase; HRP, horseradish peroxidase; *o*-DD, *o*-dianisidine; ROS, reactive oxygen species; SOD, superoxide dismutase

Address all correspondence to: Dr. Charles L. Limoli, Department of Radiation Oncology, Radiation Oncology Research Laboratory, University of California, 1855 Folsom Street, MCB-200, San Francisco, CA 94103-0806, USA. E-mail: limoli@itsa.ucsf.edu

[1]This work was supported by a grant from the American Cancer Society (RPG-00-036-01-CNE) to C. L. L.
Received 4 March 2003; Accepted 14 May 2003.

induced by X-rays, providing further evidence that oxidative stress is involved in the regulation of genome stability [16]. The genetic disorder Fanconi anemia (FA) provides an example where cancer predisposition and genomic instability are associated with a defect in the metabolism of ROS that leads to an elevated state of oxidative stress [17–20]. Although the relationship between oxidative stress and the other defects characterizing FA is unclear at present, evidence does suggest that conditions of chronic *versus* acute oxidative stress may contribute to the development and/or maintenance of genomic instability.

In an effort to understand the biochemical basis of genomic instability, we have subjected GM10115 cells to conditions of chronic oxidative stress. Here we report our findings that implicate oxidative stress as a biochemical mechanism capable of inducing a high incidence of genomic instability.

Materials and Methods

Cell Culture

The human–hamster hybrid line GM10115 was used in all studies. GM10115 cells contain 22 to 24 hamster chromosomes and a single copy of human chromosome 4 that serves as a marker to monitor chromosomal instability following exposure to genotoxic agents [6]. Cells were grown in humidified incubators at 34°C with 5% CO_2, and maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.2 mM L-proline, and 1.5 μg/ml Fungizone (GIBCO amphotericin B+1.5 μg/ml deoxycholic acid).

Chronic Treatments of Oxidative Stress

Oxidative stress was administered through the use of hydrogen peroxide. Cells were subjected to two different treatment regimes involving this oxidizing agent. One treatment involved direct addition of hydrogen peroxide to the culture medium, whereas the other treatment involved the use of glucose oxidase (GO) added to the medium to generate hydrogen peroxide enzymatically. The duration of each treatment was 1 hour and it was administered daily in serum-free medium (50 ml, high glucose ~2 mM). Cells were treated with 25, 50, 100, or 200 μM H_2O_2 (Sigma, St. Louis, MO) or 5, 10, 25, or 50 mU/ml GO (type VII; Sigma). Daily exposure of cultures to 200 μM H_2O_2 were limited to 10 days; all other treatments were carried out over the course of 30 days. Before and after treatment with H_2O_2, cultures were rinsed twice in isotonic phosphate-buffered saline (PBS). Cultures subjected to chronic oxidative stress were not passaged until the penultimate treatment. Expanded cultures were treated as before and provided the cells necessary for the determination of multiple endpoints. Following successive H_2O_2 treatments, cells were harvested and analyzed for first division aberrations and clonogenic survival, or reseeded for colony formation and the isolation of colonies for the assessment of chromosomal instability.

Determination of H_2O_2 Concentration

A colorimetric assay was used to measure H_2O_2. The modified assay adapted from Nowak [21] is essentially as described by Salazar and Houten [22], and measures the enzymatic conversion of a redox-sensitive substrate. The assay is based on the ability of type II horseradish peroxidase (HRP) to use H_2O_2 in the conversion of *o*-dianisidine (*o*-DD) to a colored substrate. Reactions were initiated by adding 200 μl of the sample to 800 μl of 1× PBS containing 80 μg/ml *o*-DD (Sigma) and 5 U of HRP (Sigma). Reactions were then placed at 37°C for 1 hour before measuring absorbance at 470 nm. Stock solutions of *o*-DD (Sigma) and HRP (Sigma) were made fresh daily in double distilled water and concentrations of all H_2O_2 stocks were determined spectrophotometrically (230 nm, ε=81/cm/mol). For standard curves, samples consisted of H_2O_2 or GO diluted in serum-free media.

Cytogenetics—First Division Aberrations

To determine the extent of chromosomal damage induced directly after chronic oxidative stress, cultures were analyzed for the presence of aberrations involving human chromosome 4. One day following the final H_2O_2 treatment, one set of cultures was treated with Colcemid (2×10^{-7} M) and processed for the preparation of metaphase spreads as described previously [6]. Slides containing metaphases were subjected to fluorescence *in situ* hybridization (FISH) using a probe derived from a whole human chromosome 4–specific library and analyzed by fluorescence microscopy. When possible, the analysis of rearrangements was based on a minimum of 1000 metaphases; however, the poor condition of cultures after certain conditions (200 μM H_2O_2, 50 mU/ml GO) limited the yield of quality metaphases for analysis. Chromosome-type aberrations were scored using the PAINT terminology as described previously [23].

Survival and Isolation of Colonies for the Determination of Chromosomal Instability

One day after the final treatment with H_2O_2, another set of cultures was harvested and cells were plated for the determination of survival by clonogenic assay. Multiple plates and cell densities were seeded to optimize the number of colonies available for scoring and isolation. Plates containing over 10 colonies were stained with 0.1% crystal violet in 25% ethanol and scored exclusively for the assessment of surviving fraction. Plates containing less than 10 colonies were selected for the isolation of individual clones destined for expansion and cytogenetic analysis. Clones were picked with sterile trypsin-soaked swabs and expanded to mass population for freezing and the preparation of metaphase spreads.

Cytogenetics—Chromosomal Instability

Colonies derived from single progenitor cells surviving chronic oxidative stress were picked at random and expanded to mass population for the analysis of chromosomal instability by FISH. Chromosomal instability following chronic oxidative stress was assessed in over 180

independent clones, and the analysis of individual clones was based on a minimum of 100 metaphases and encompassed over 36,000 metaphases total. Clones exhibiting a minimum of three subpopulations containing distinct types of chromosome aberrations that represented at least 5% of all metaphases scored were classified as chromosomally unstable. This rigorous criterion effectively minimizes false positives and has been found to be a reliable measure of chromosomal instability [24].

Results

Measurement of H_2O_2

To properly assess the conditions for the exposure of cells to chronic oxidative stress, it was necessary to accurately measure the level of H_2O_2 in the culture medium. This was performed using a modified version of an assay that uses HRP to enzymatically convert *o*-DD to a colored substrate in the presence of H_2O_2. Under the appropriate conditions, this reaction depends linearly on the concentration of H_2O_2 and can be used to generate a standard curve as that shown in Figure 1. The plots shown in Figure 1 clearly demonstrate that the conversion of *o*-DD is dependent upon the presence of HRP, and that the response of this assay is linear over a wide range of H_2O_2 concentrations (10–200 μM). The curve generated in the presence of 5 U/ml HRP was derived from three independent measurements and was used to determine the level of H_2O_2 under various culture conditions.

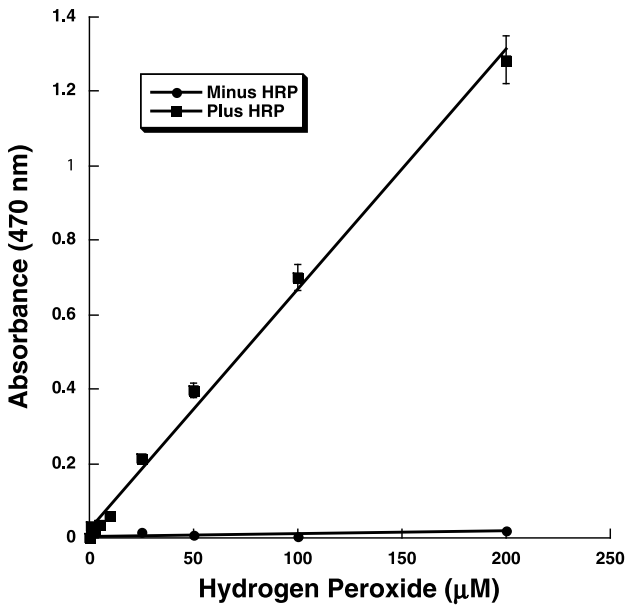

Figure 1. *Standard curve for the measurement of H_2O_2. The 1.0, 10, and 100 mM H_2O_2 stock solutions were prepared and the actual concentration of these solutions was calculated based on its absorbance at 230 nm (ε=81/cm/mol). Aliquots of H_2O_2 (200 μl) were added to 800 μl of PBS containing o-DD (80 μg/ml) and 5 U of HRP (squares) or no HRP (circles). Samples were incubated at 37°C for 1 hour before measuring absorbance at 470 nm. Plots shown are the linear regression fits through all data derived from three independent experiments (±SD).*

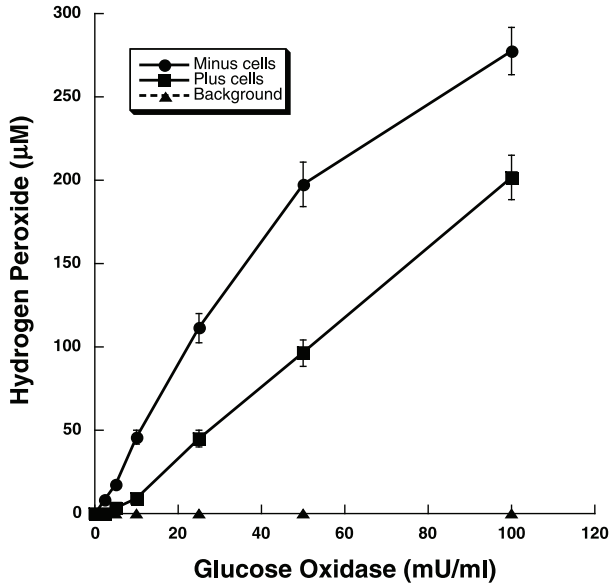

Figure 2. *Generation of H_2O_2 by GO. GO treatment for 1 hour was made in serum-free DMEM in the presence (~ 1×10^6, squares) or absence (circles) of cells. The background generation of H_2O_2 in cells was also determined (triangles). Following a range of GO treatments, samples were removed and assayed for H_2O_2 using the o-DD/HRP assay. Data shown were averaged from duplicate samples taken from each of two independent experiments (±SD).*

Generation of H_2O_2 by GO

To assess the ability of GO to generate H_2O_2, various concentrations of GO (2.5–100 mU/ml) were dissolved in 5 ml of serum-free medium and added to 60-mm plates with and without cells. One day prior to assay, 1×10^6 cells were seeded into plates that were rinsed prior to the addition of GO. All plates receiving GO were incubated for 1 hour at 37°C. Aliquots (200 μl) were removed after 1 hour and assayed for H_2O_2 as described above. The results of these experiments are shown in Figure 2 and indicate the efficiency with which GO is able to generate H_2O_2 under the stated conditions. The production of H_2O_2 is observed to be linearly proportional to the amount of GO. In the absence of cells, a five-fold increase in the concentration of GO (10–50 mU/ml) leads to a four-fold increase in the concentration of H_2O_2 (50–200 μM); the production of H_2O_2 is less efficient when the concentration of GO exceeds 50 mU/ml (Figure 2, *circles*). In the presence of cells, the level of H_2O_2 in the medium is reduced but is still observed to increase linearly (10–200 μM) with GO concentration (10–100 mU/ml). The metabolic activity of ~1.5 million cells is sufficient to reduce the level of H_2O_2 by ~50% when the concentration of GO is less than 50 mU/ml (Figure 2, *squares*). These experiments demonstrate the utility of the GO system for generating H_2O_2 in culture.

Kinetics of H_2O_2 Metabolism

Time course studies were undertaken to establish the steady state levels of H_2O_2 in culture. Using the assay format described above, H_2O_2 levels were observed to rise rapidly (~40 μM within 5 minutes) in the presence of 25 mU/ml GO (Figure 3). In the absence of cells, H_2O_2 increased linearly

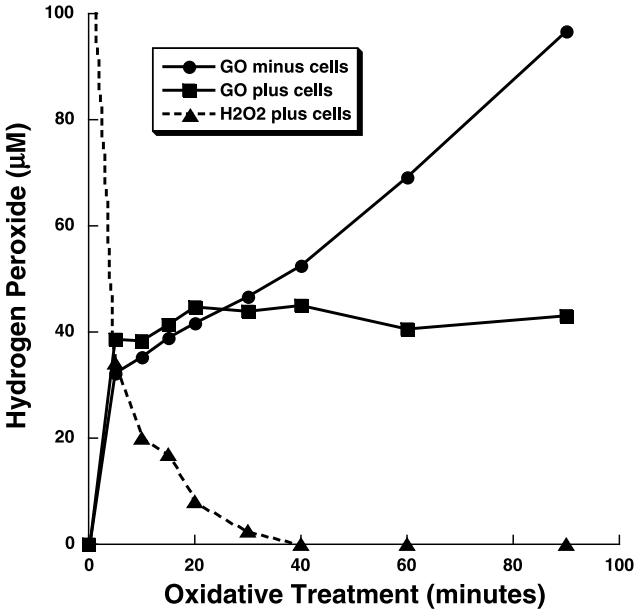

Figure 3. *Kinetics of H_2O_2 production and metabolism. GO (25 mU/ml) was added to serum-free DMEM in the presence (~ 1×10^6, squares) or absence (circles) of cells. H_2O_2 (200 μM) was also added to cultures of 1×10^6 cells in serum-free DMEM as a comparison (triangles). Following the addition of GO or H_2O_2, aliquots were removed over the course of an hour and assayed for H_2O_2 content using o-DD and HRP. Plots shown were averaged from duplicate samples taken from a typical experiment.*

during the course of treatment, reaching ~100 μM by 60 minutes (Figure 3, *circles*), a value comparable to that shown in Figure 2 (*circles*). In the presence of cells, an equilibrium that resulted in a steady state level of ~40 μM H_2O_2 throughout the duration of the treatment was achieved (Figure 3, *squares*). When H_2O_2 was added directly to cultures, cellular metabolism rapidly depleted H_2O_2 levels to background within 40 minutes (Figure 3, *triangles*). These experiments illustrate that cells deplete exogenously added H_2O_2 rapidly, and that steady state levels can only be maintained when a H_2O_2-generating system is used. Thus, the two treatment regimes used to expose cells to chronic oxidative stress exhibit markedly different kinetic profiles for the consumption of H_2O_2.

Chronic Exposure Conditions

Cultures subjected to chronic oxidative stress through acute addition of H_2O_2 or through treatment with GO were assayed periodically for H_2O_2 levels over the course of treatment. Triplicate samples were removed for analysis on the day of assay and at the end of each specific 1-hour treatment. Cultures tested in parallel on days 5, 15, and 30 indicate that H_2O_2 was produced in a manner dependent upon the concentration of GO (Figure 4). Modest fluctuations in H_2O_2 levels were observed for a given treatment over 30 days. These fluctuations were due to unavoidable variability in the day-to-day treatments and the expected variability in cell numbers resulting from the dynamic turnover of cells cultured continuously over 1 month. As opposed to cultures treated with GO, cultures degraded an acute bolus of H_2O_2 to undetectable levels by 1 hour—data consistent with the rapid elimination of H_2O_2 shown in Figure 3 (*dashed line*).

Cell Survival Following Chronic Oxidative Stress

Cells were harvested for the assessment of clonogenic survival 1 day following the final oxidizing treatment. Surviving fraction was normalized to control plating efficiency (76%) and expressed as a function of GO or H_2O_2 concentration (Figure 5). Chronic treatments lasting 30 days were

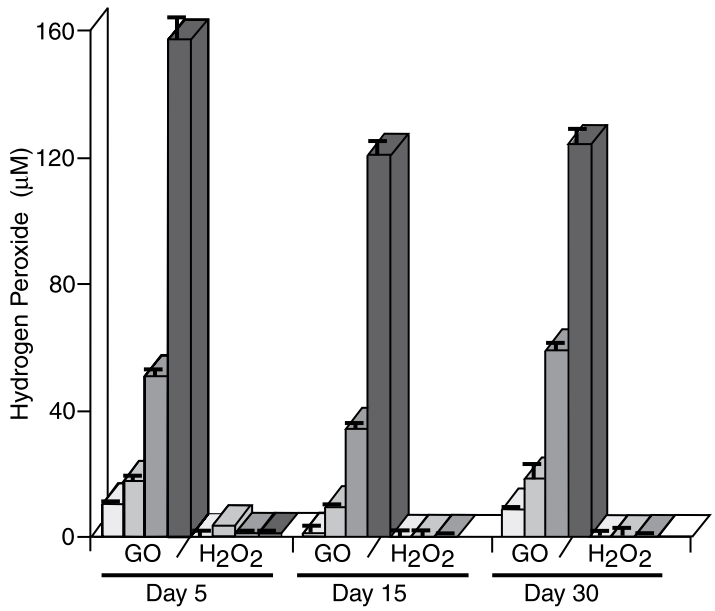

Figure 4. *Chronic levels of H_2O_2 exposure. GM10115 cells subjected to chronic oxidative stress were assayed periodically for the determination of H_2O_2 levels in culture. Triplicate samples removed on days 5, 15, and 30 were analyzed for H_2O_2 content by the o-DD/HRP assay, and values were converted to H_2O_2 concentration using the standard curve shown in Figure 1. For each day, increased shading of individual bars (from left to right) corresponds to 5, 10, 25, and 50 mU/ml GO or 25, 50, 100, and 200 (day 5 only) μM H_2O_2. Bar charts indicate the average of triplicate measurements (±SD).*

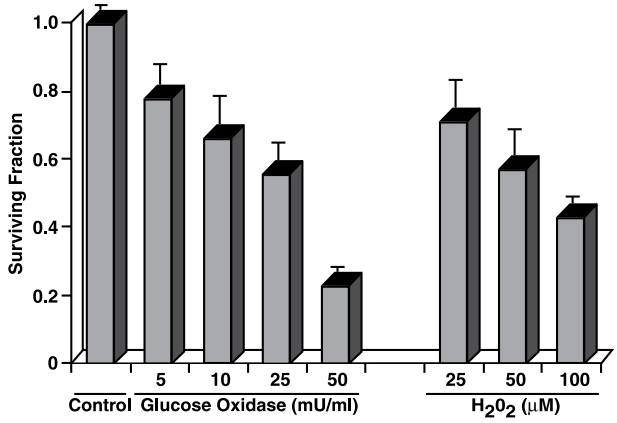

Figure 5. *Cell survival after chronic oxidative stress. GM10115 cells subjected to 30 consecutive days of GO or H_2O_2 exposure were plated for the determination of clonogenic survival. Surviving fraction was normalized to sham-treated controls set to unity. Data represent the average of three independent measurements (±SD).*

not found to be markedly toxic. Surviving fraction was reduced from 0.78 to 0.23 as the concentration of GO increased from 5 to 50 mU/ml, respectively. Increasing the amount of H_2O_2 added exogenously from 25 to 100 μM dropped survival by only 40%. The relatively modest toxicity observed after chronic oxidative stress treatments is in contrast to our previous studies noting a higher level of toxicity associated with the acute treatment of GM10115 cells with superoxide or H_2O_2 [6,7].

Early Division Aberrations

Cells were also analyzed for chromosomal rearrangements directly after the last oxidative treatment. First and second division aberrations involving human chromosome 4 were used to gauge the clastogenic properties of chronic oxidative stress treatments. The fraction of metaphases containing human chromosome 4 aberrations or kickouts trended upward with increasing GO or H_2O_2 concentration (Table 1). None of the treatments led to more than 2% aberrant metaphases except 100 μM H_2O_2, which produced a yield of 4.3% aberrant metaphases (Table 1). Similarly, the number of metaphases devoid of the human chromosome did not exceed 5% for any of the treatments except 100 μM H_2O_2, which resulted in the loss of human chromosome 4 from 11% of the metaphases scored (Table 1). Further visual analysis of slides revealed the presence of metaphases that possessed multiple complex aberrations (data not shown). These metaphases were observed at GO levels of 10 and 25 mU/ml, and at H_2O_2 levels of 50 and 100 μM. Most of these metaphases contained multiple aberrations (>5) characterized by complex translocations, acentric fragments, and a smaller proportion of dicentrics and rings. Metaphases exhibiting multiple aberrations were quantitatively insignificant (<0.1%), but did suggest that a small but detectable fraction of cells exposed to chronic oxidative stress had begun to exhibit indications of chromosomal instability. Early division metaphases derived from cells exposed to 50 mU/ml GO or 200 μM H_2O_2 were of insufficient quality for cytogenetic analysis.

Table 1. Chromosomal Aberrations Found Directly After Chronic Oxidative Stress.

Treatment*	Percentage of Aberrant Metaphases†	Percentage of metaphases without human chromosome 4‡
Controls	0.2	0.8
H_2O_2, 25 μm	0.3	1.6
H_2O_2, 50 μm	0.4	4.6
H_2O_2, 100 μm	4.3	11.3
GO, 5 mU/ml	1.0	1.3
GO, 10 mU/ml	2.0	2.7
GO, 25 mU/ml	1.6	4.0

*One thousand total metaphases scored for each treatment.
†Percentage of metaphases containing rearrangements involving human chromosome 4.
‡Percentage of metaphases not containing human chromosome 4.

Chromosomal Instability Resulting from Chronic Oxidative Stress

Colonies surviving exposure to chronic oxidative stress were clonally expanded for the analysis of potential chromosomal instability. Based on the low frequency of metaphases containing first division aberrations and the infrequent occurrence of metaphases containing multiple aberrations, we chose to focus our analysis on those clones surviving the highest levels of oxidative stress (i.e., 50 mU/ml GO or 100 and 200 μM H_2O_2). This approach was designed to optimize the detection of chromosomally unstable clones because past experience has found that encountering this phenotype among randomly selected clones is exceedingly rare at the levels of survival shown in Figure 5 [6]. Somewhat unexpectedly, cytogenetic analysis of 182 clones representing over 36,000 metaphases indicates a relatively high efficiency with

Table 2. Chromosomal Instability in Clones Subjected to Chronic Oxidative Stress.

Treatment	Number of Clones	Number of Metaphase Subpopulations*	Percentage of Metaphases†
Controls	44	0–3	0–3
H_2O_2, 100 μm	31	0–2	0–5.5
	2	1–2	97–100
	6	3–4	1.5–3.5
	5	**3–5**	5–18
H_2O_2, 200 μm	35	0–4	0–3.5
	1	1	61
	7	**3–5**	6.5–10.5
	3	**7–10**	52–75
GO, 50 mU/ml	39	0–3	0–4
	7	**3–6**	5–23
	2	**7–8**	21–100

Boldface indicates chromosomally unstable clones, as defined by those clones containing three or more abnormal metaphase subpopulations comprising at least 5% of all metaphases scored.
*Number of different abnormal metaphase subpopulations showing distinct types of chromosome aberrations.
†Percentage of metaphases scored showing chromosomal aberrations.

which chronic oxidative stress elicits chromosomal instability (Table 2). The fraction of clones exhibiting chromosomal instability (i.e., those clones containing greater than three or more aberrant subpopulations comprising at least 5% of the metaphases analyzed) after exposure to 100 or 200 μM H_2O_2, or 50 mU/ml GO was found to be 5/44 (11%), 10/46 (22%), and 9/48 (19%) respectively. Using GO treatment as an example, seven clones exhibited three to six different types of aberrations within 5% to 23% of the metaphases, whereas two clones had seven or eight different types of aberrations within 21% or all of the metaphases scored. All 44 control clones subjected to the same experimental manipulations but receiving no GO or H_2O_2 had zero to three aberrant subpopulations involving 0% to 3% of the metaphases and were, therefore, not chromosomally unstable.

Discussion

Earlier studies have demonstrated a variable degree of efficiency with which DNA-damaging agents were able to elicit chromosomal instability [6,7]. Although the different spectrum of DNA damage types provides some explanation for this variability, it was unlikely that the inefficient induction of chromosomal instability by these acute oxidizing treatments was due to an insufficient level of DNA damage because clonogenic survival was reduced by 2 to 4 logs kill [6,7]. However, the persistence of oxidative stress observed in clones that did exhibit chromosomal instability [7] and the amplification of redox-sensitive genes in cells exposed to chronic oxidative stress [14] suggested that chronic, as opposed to acute, oxidizing treatments might lead to a higher incidence of chromosomal instability.

To explore this possibility, we utilized two treatment regimes for subjecting cells to chronic oxidative stress: one approach involved the addition of H_2O_2 directly to the culture medium, whereas the other approach used the enzyme GO to generate H_2O_2 when added to the culture medium. Two separate approaches were used because the treatment profiles of H_2O_2 exposure in culture differ so markedly. The rapid metabolism of exogenously added H_2O_2 exposes cells to a steep gradient of H_2O_2 concentrations, whereas GO leads to a more steady state level of H_2O_2 (Figure 3). Oxidizing treatments were carried out for 30 days, except those involving 200 μM H_2O_2, because past studies have shown that 1 month is generally required to detect chromosomal instability in individual clones isolated and expanded after the initial induction of DNA damage.

Cultures subjected to chronic oxidative stress exhibited some expected variations in H_2O_2 levels because it was difficult to control the absolute number of cells cultured over the duration of the chronic treatments. Cells were also in a relatively high state of confluency (>90%) during most of the chronic treatments, as repopulation continually balanced the cell kill resulting from daily administration of H_2O_2. Density-inhibited cell growth may underlie the lower toxicity of chronic *versus* acute oxidative stress in GM10115 cells because the fraction of cells actively proliferating during oxidant exposure was reduced under chronic conditions [25–27]. The relatively modest toxicity of chronic treatments (20–80% kill) may also reflect compensatory responses developed during chronic exposures that bolster the selective pressure for propagating cells having an increased resistance to H_2O_2.

The lack of marked toxicity after chronic oxidative stress was accompanied by the absence of numerous first division aberrations. Cells analyzed within the first two cell divisions after chronic oxidative exposures were found to exhibit a minimum (<5%) of chromosomal aberrations involving the human chromosome. Quantitatively, these aberration and survival (>20%) levels corroborate our earlier measurements finding a linear relationship between cell survival and the yield of aberrations involving human chromosome 4 [16].

Although initial aberration levels are reliable predictors of clonogenic survival, their utility in forecasting the incidence of chromosomal instability is less certain. Chromosome aberrations detected shortly after damaging treatments reflect the clastogenic properties of a particular agent, and are generally not considered to be the result of chromosomal instability [28]. Nonetheless, the frequency of aberrant metaphases may provide a marker for the number of cells having an increased likelihood of developing chromosomal instability. In one treatment (100 μM H_2O_2), the frequency of metaphases exhibiting first division aberrations (4.3%) was less than the frequency of clones exhibiting chromosomal instability (11%). Furthermore, a linear extrapolation of first division aberration yields from lower H_2O_2 or GO levels still underestimates the incidence of chromosomal instability observed at 200 μM H_2O_2 (22%) or 50 mU/ml GO (19%). The extremely low occurrence (<0.1%) of metaphases exhibiting multiple complex aberration types at the first division suggests, too, that chromosomal instability was not yet fully developed immediately following chronic treatments. Although it is tempting to speculate that chronic oxidative stress primed a latent program for activation during clonal expansion to elicit instability, there are caveats to this interpretation as well. Significantly, first division aberration yields reported (Table 1) are underestimates of total aberration yields because the human chromosome comprises ~5% of the GM10115 genome [29]. Any further evaluation of this possibility was also complicated by the difficulty of obtaining quality metaphases for early cytogenetic analysis after 200 μM H_2O_2 and 50 mU/ml GO treatments.

Present data show that chronic oxidative stress elicits a high frequency of chromosomal instability when compared to acute oxidative stress. In prior studies using GM10115 cells, chromosomal instability was not observed in over 80 clones surviving acute treatments of H_2O_2 or superoxide (by xanthine/xanthine oxidase) that resulted in 2 to 4 logarithmic orders of cell kill [6,7]. In contrast, present studies have demonstrated that chromosomal instability is observed in 10% to 20% of clones surviving chronic exposure to 100 μM, 200 μM H_2O_2, or 50 mU/ml GO. Despite the different steady state levels of H_2O_2 arising from the addition of GO *versus* H_2O_2, both chronic treatments were relatively effective at producing chromosomally unstable clones. Although the reasons for this are uncertain, the marked increase in

efficiency with which chronic oxidative stress induces chromosomal instability does indicate that repeated disruptions to the redox environment of a cell are sufficient to compromise genomic integrity.

The current study was designed to assess the impact of ROS on cells rather than the origin or source of cellular ROS. Exogenous H_2O_2 may modulate extracellular redox processes that impact cellular physiology. The importance of extracellular SOD [30,31] and NADH oxidases [32] in regulating multiple pathways supports the physiological relevance of the extracellular redox environment. Observations suggesting an involvement of ROS in mediating the interactions between undamaged cells and those damaged by irradiation [33,34] implicate a role for exogenous ROS in the stress response of cells. These so-called "bystander effects" have been hypothesized to propagate the abnormal phenotypes associated with genomic instability [35].

Cancer cells exhibit multiple endpoints of genomic instability [2–4] and a loss of regulation between glycolytic metabolism and mitochondrial respiration [36,37]. The increased dependence upon glycolysis shown by most cancer cells [36,37] may underlie their sensitivity to oxidative stress induced by glucose deprivation [38–40]. Many of the signaling and gene expression pathways believed important in neoplastic transformation are activated under conditions of oxidative stress and glucose deprivation [38–40]. Therefore, it seems reasonable that under certain situations, the tumor microenvironment may be subject to chronic oxidative stress that promotes the accumulation of ROS. Cancer cells have been shown to produce elevated H_2O_2 [41] and glucose metabolism appears to be integrally related to the metabolic detoxification of intracellular ROS [42]. Consequently, tumor cells may well increase their metabolism of glucose to compensate for increased ROS production caused by a defect in mitochondrial respiration [40]. Our recent findings demonstrating that chromosomal instability is associated with a persistent increase in intracellular ROS and an increased number of dysfunctional mitochondria support this idea further [43]. Although it remains to be determined how chronic oxidative stress impacts tumorigenesis *in vivo*, present data suggest that metabolic and/or environmental conditions that might lead to a persistent elevation in oxidative species can exacerbate the onset of genomic instability.

References

[1] Fearon ER, and Vogelstein B (1990). A genetic model for colorectal tumorigenesis. *Cell* **61,** 759–67.

[2] Fearon ER (1997). Human cancer syndromes: clues to the origin and nature of cancer. *Nature* **278,** 1043–1049.

[3] Morgan WF, Day JP, Kaplan MI, McGhee EM, and Limoli CL (1996). Genomic instability induced by ionizing radiation. *Radiat Res* **146,** 247–58.

[4] Nowell PC (1976). The clonal evolution of tumor cell populations. *Science* **194,** 23–28.

[5] Loeb LA (1991). Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res* **51,** 3075–3079.

[6] Limoli CL, Kaplan MI, Phillips JW, Adair GM, and Morgan WF (1997). Differential induction of chromosomal instability by DNA strand-breaking agents. *Cancer Res* **57,** 4048–4056.

[7] Limoli CL, Hartmann A, Shephard L, Yang CR, Boothman DA, Bartholomew J, and Morgan WF (1998). Apoptosis, reproductive failure, and oxidative stress in Chinese hamster ovary cells with compromised genomic integrity. *Cancer Res* **58,** 3712–718.

[8] Voet D, Voet JG, and Pratt CW (1999). *Fundamentals of Biochemistry: Electron Transport and Oxidative Phosphorylation (Chapter 17).* John Wiley and Sons, Inc., New York. pp. 492–525.

[9] Boveris A (1977). Mitochondrial production of superoxide radical and hydrogen peroxide. *Adv Exp Med Biol* **78,** 67–82.

[10] Boveris A, and Cadenas E (1982). In *Superoxide Dismutase: Vol. II, Production of Superoxide Radicals and Hydrogen Peroxide in Mitochondria.* LW Oberly, Ed. CRC Press, Boca Raton, FL. pp. 15–30.

[11] Nohl H, and Jordan W (1986). The mitochondrial site of superoxide production. *Biochem Biophys Res Commun* **138,** 533–39.

[12] Ames BN, Shigenaga MK, and Hagen TM (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci USA* **90,** 7915–922.

[13] Lindahl T (1990). Repair of intrinsic DNA lesions. *Mutat Res* **238,** 305–311.

[14] Hunt CR, Sim JE, Sullivan SJ, Featherstone T, Golden W, VonKappHerr C, Hock RA, Gomez RA, Parsian AJ, and Spitz DR (1998). Genomic instability and catalase gene amplification induced by chronic exposure to oxidative stress. *Cancer Res* **58,** 3986–992.

[15] Clutton SM, Townsend KMS, Walker C, Ansell JD, and Wright EG (1996). Radiation-induced genomic instability and persisting oxidative stress in primary bone marrow cultures. *Carcinogenesis* **17,** 1633–639.

[16] Limoli CL, Kaplan MI, Giedzinski E, and Morgan WF (2001). Attenuation of radiation-induced genomic instability by free radical scavengers and cellular proliferation. *Free Radic Biol Med* **31,** 10–19.

[17] Ruppitsch W, Meisslitzer C, Weirich-Schwaiger H, Klocker H, Scheidereit C, Schweiger M, and Hirsch-Kauffmann M (1997). The role of oxygen metabolism for the pathological phenotype of Fanconi anemia. *Hum Genet* **99,** 710–19.

[18] Kruyt FA, Hoshino T, Liu JM, Joseph P, Jaiswel AK, and Youssoufian H (1998). Abnormal microsomal detoxification implicated in Fanconi anemia group C by interaction of the FAC protein with NADPH cytochrome *P*450 reductase. *Blood* **92,** 3050–3056.

[19] Cumming RC, Lightfoot J, Beard K, Youssoufian H, O'Brien PJ, and Buchwald M (2001). Fanconi anemia group C protein prevents apoptosis in hematopoietic cells through redox regulation of GSTP1. *Nat Med* **7,** 814–20.

[20] Futaki M, Igarashi T, Watanabe S, Kajigaya S, Tatsuguchi A, Wang J, and Liu JM (2002). The FANCG Fanconi anemia protein interacts with CYP2E1: possible role in protection against oxidative DNA damage. *Carcinogenesis* **23,** 67–72.

[21] Nowak D (1990). Hydrogen peroxide release from human polymorphonuclear leukocytes measured with horseradish peroxidase and *o*-dianisidine. Effect of various stimulators and cytochalasin B. *Biomed Biochim Acta* **49,** 353–62.

[22] Salazar JJ, and Houten BV (1997). Preferential mitochondrial DNA injury caused by glucose oxidase as a steady generator of hydrogen peroxide in human fibroblasts. *Mutat Res* **385,** 139–49.

[23] Tucker JD, Morgan WF, Awa AA, Bauchinger M, Blakey D, Cornforth MN, Littlefield LG, Natajaran AT, and Shasserre C (1995). A proposed system for scoring structural aberrations detected by chromosome painting. *Cytogenet Cell Genet* **68,** 211–21.

[24] Limoli CL, Corcoran JJ, Milligan JR, Ward JF, and Morgan WF (1999). Critical target and dose and dose-rate responses for the induction of chromosomal instability by ionizing radiation. *Radiat Res* **151,** 677–85.

[25] Little JB (1969). Repair of sublethal and potentially lethal radiation damage in plateau phase cultures of human cells. *Nature* **224,** 804–806.

[26] Cornforth MN, and Bedford JS (1987). A quantitative comparison of potentially lethal damage repair and the rejoining of interphase chromosome breaks in low passage normal human fibroblasts. *Radiat Res* **111,** 385–405.

[27] Iliakis GE, and Okayasu R (1990). Radiosensitivity throughout the cell cycle and repair of potentially lethal damage and DNA double-strand breaks in an X-ray–sensitive CHO mutant. *Int J Radiat Biol* **57,** 1195–211.

[28] Marder BA, and Morgan WF (1993). Delayed chromosomal instability induced by DNA damage. *Mol Cell Biol* **13,** 6667–677.

[29] Day JP, Limoli CL, and Morgan WF (1998). Recombination involving interstitial telomere repeat-like sequences promotes chromosomal instability in Chinese hamster cells. *Carcinogenesis* **19,** 259–66.

[30] Levin ED, Brady TC, Hochrein EC, Oury TD, Jonsson LM, Marklund SL, and Crapo JD (1998). Molecular manipulations of extracellular

superoxide dismutase: functional importance for learning. *Behav Genet* **28,** 381–90.
[31] Fukai T, Folz RJ, Landmesser U, and Harrison DG (2002). Extracellular superoxide dismutase and cardiovascular disease. *Cardiovasc Res* **55,** 239–49.
[32] Berridge MV, and Tan AS (2000). High-capacity redox control at the plasma membrane of mammalian cells: trans-membrane, cell surface, and serum NADH-oxidases. *Antioxid Redox Signal* **2,** 231–342.
[33] Iyer R, and Lehnert BE (2000). Factors underlying the cell growth-related bystander responses to alpha particles. *Cancer Res* **60,** 1290–298.
[34] Iyer R, and Lehnert BE (2002). Low dose, low-LET ionizing radiation-induced radioadaptation and associated early responses in unirradiated cells. *Mutat Res* **503,** 1–9.
[35] Morgan WF, Hartmann A, Limoli CL, Nagar S, and Ponnaiya B (2002). Bystander effects in radiation-induced genomic instability. *Mutat Res* **504,** 91–100.
[36] Weber G (1977). Enzymology of cancer cells (Part I). *N Engl J Med* **296,** 486–92.
[37] Weber G (1977). Enzymology of cancer cells (Part II). *N Engl J Med* **296,** 541–51.
[38] Lee YJ, Galoforo SS, Berns CM, Chen JC, Davis BH, Sim JE, Corry PM, and Spitz DR (1998). Glucose deprivation-induced cytotoxicity and alterations in mitogen-activated protein kinase activation are mediated by oxidative stress in multidrug-resistant human breast carcinoma cells. *J Biol Chem* **273,** 5294–299.
[39] Blackburn RV, Spitz DR, Liu X, Galoforo SS, Sim JE, Ridnour LA, Chen JC, Davis BH, Corry PM, and Lee YJ (1999). Metabolic oxidative stress activates signal transduction and gene expression during glucose deprivation in human tumor cells. *Free Radic Biol Med* **26,** 419–30.
[40] Spitz DR, Sim JE, Ridnour LA, Galoforo SS, and Lee YJ (2000). Glucose deprivation-induced oxidative stress in human tumor cells. A fundamental defect in metabolism? *Ann NY Acad Sci* **899,** 349–62.
[41] Szatrowski TP, and Nathan CF (1991). Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* **51,** 794–98.
[42] Averill-Bates DA, and Przybytkowski E (1994). The role of glucose in cellular defences against cytotoxicity of hydrogen peroxide in Chinese hamster ovary cells. *Arch Biochem Biophys* **312,** 52–58.
[43] Limoli C, Giedzinski E, Morgan W, Swarts S, Jones G, and Hyun W (2003). Persistent oxidative stress in chromosomally unstable cells. *Cancer Res* **63,** 3107–111.