DNA damage in cells exhibiting radiation-induced genomic instability

Deborah J. Keszenman$^{1,2}$, Lucia Kolodiuk$^3$, and Janet E. Baulch$^4$

$^1$ Biosciences Department, Brookhaven National Laboratory, 50 Bell Avenue, Upton, NY 11973, USA
$^2$ Laboratory of Medical and Environmental Radiobiology, Biophysical Chemistry Group, Department of Biological Sciences, CENUR del Noroeste, UdelaR, Rivera 1350, Salto 50000, Uruguay
$^3$ 107-112 CMM/BLL, Stony Brook University, Stony Brook, NY 11794, USA
$^4$ Department of Radiation Oncology, University of California, Medical Sciences I, B149, Irvine, CA 92697, USA

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DNA Damage in Cells Exhibiting Radiation Induced Genomic Instability

Deborah J. Keszenman 1,2*, Lucia Kolodiuk 3 and Janet E. Baulch 4*

1 Biosciences Department, Brookhaven National Laboratory, 50 Bell Avenue, Upton, NY 11973, USA
2 Laboratory of Medical and Environmental Radiobiology, Biophysical Chemistry Group, Department of Biological Sciences, CENUR del Noroeste, UdelaR, Rivera 1350, Salto, 50000, Uruguay; keszenman@unorte.edu.uy
3 Undergraduate Biology, 107-112 CMM/BLL, Stony Brook University, Stony Brook, NY 11794, USA
4 Department of Radiation Oncology, University of California, Medical Sciences I, B149, Irvine, CA 94967, USA; E-Mail: jbaulch@uci.edu

* Authors to whom correspondence should be addressed; E-Mail: jbaulch@uci.edu; Tel.: 1(949)824-7396; Fax: 1(949)824-3566; keszenman@unorte.edu.uy; Tel: 1(631) 681-4098

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Abstract

Cells exhibiting radiation induced genomic instability exhibit varied spectra of genetic and chromosomal aberrations. Even so, oxidative stress remains a common theme in the initiation and/or perpetuation of this phenomenon. Isolated oxidatively modified bases, abasic sites, DNA single strand breaks, and clustered DNA damage are induced in normal mammalian cultured cells and tissues due to endogenous reactive oxygen species generated during normal cellular metabolism in an aerobic environment. While sparse DNA damage may be easily repaired, clustered DNA damage may lead to persistent cytotoxic or mutagenic events that can lead to genomic instability. In this study we tested the hypothesis that DNA damage signatures characterized by altered levels of endogenous, potentially mutagenic, types of DNA damage and chromosomal breakage are related to radiation induced genomic instability and persistent oxidative stress phenotypes observed in the chromosomally unstable progeny of irradiated cells. The measurement of oxypurine, oxypyrimidine, and abasic site endogenous DNA damage showed differences in non-DSB clusters among the three of the four unstable clones evaluated as compared to genomically stable clones and the parental cell line. Three of the four unstable clones also had increased levels of DSB clusters. The results of this study demonstrate that each unstable cell line has a unique spectrum of persistent damage and lead us to speculate that alterations in DNA damage signaling and repair may be related to the perpetuation of genomic instability.
1. Introduction

Genomic instability encompasses diverse changes such as reduced subcloning efficiencies, karyotypic heterogeneity, chromosomal modifications, micronucleus formation, changes in mutation rates, gene amplifications and/or microsatellite instability [1, 2] and it has been proposed as a hallmark of cancer [3]. Several studies have demonstrated that genomic instability can be induced by oxidative stress inducing genotoxic agents [1, 4, 5, 6, 7, 8, 9]. Furthermore, oxidative stress may cause a persistent alteration in the maintenance of genomic integrity as shown in studies using the human–hamster hybrid GM10115 cell line and derived clones. These studies have established a functional link among radiation exposure, genomic instability and oxidative stress [4, 5].

Normal cellular metabolism in an aerobic environment is well established as the source of endogenous reactive oxygen species (ROS), which accounts for background levels of oxidative DNA damage. Isolated oxidatively modified bases (oxypurines and oxypyrimidines), abasic sites, DNA single strand breaks as well as clustered DNA damage are induced in normal mammalian cultured cell lines and tissues. Clustered DNA damage is defined as two or more strand breaks, oxidized bases or abasic sites located within one or two helical turns, or within approximately 20 base pairs [10, 11, 12, 13, 14, 15]. While oxidative DNA damage and abasic sites are predominantly repaired by the base excision repair (BER) pathway, double strand breaks (DSBs) are repaired by non-homologous end-joining (NHEJ) and/or homologous recombination (HR). HR operates mainly, but not exclusively, in S and G2/M phases of the cell cycle [16]. However, it is known that more complex clustered damage is difficult for cells to repair [10, 17, 18]. Therefore, under adverse oxidative environmental conditions or by modification of cellular repair proficiency,
the levels of endogenous damage clusters could be altered and provide a source of persistent cytotoxic or mutagenic events leading to genomic instability.

Genomic instability and carcinogenesis are both multifactorial phenomena. More than a single mechanism or change contributes to the initiation and perpetuation of genomic instability and many changes are also necessary for a normal cell to become tumorigenic. This has been demonstrated particularly well in a set of well characterized clonally expanded single cell survivors of irradiation. These genomically unstable cells are not only chromosomally unstable, but also exhibit other changes in cellular phenotype such as increased HPRT mutation frequency, increased cytosolic cytochrome c, and reduced plating efficiency [4, 5, 19, 20].

Mechanisms thought to contribute to this instability include increased cytokine secretion and oxidative stress. In particular, the CS9 and LS12 unstable clones have been shown to exhibit mitochondrial dysfunction that contributes to oxidative stress [4, 20]. Interestingly, mitochondrial dysfunction may also impact other physiological functions including antioxidant defense, and intracellular signaling [21]. These increased intracellular ROS levels may induce nuclear or mitochondrial DNA damage, as well as mitochondrial oxidative stress signaling, both affecting nuclear gene expression [22, 23]. Furthermore, several lines of evidence support the hypothesis that this feedback loop between oncogenic signal transduction cascades and mitochondrial function constitutes an important mechanism underlying carcinogenesis [22, 24, 25].

In this study we are testing the hypothesis that the unstable clones derived from the human-hamster hybrid GM10115 cell line will exhibit DNA damage signatures unique to the genomic instability phenotype. Defining these DNA damage signatures may also provide insight into the mechanisms underlying carcinogenesis.
The assessment of the different classes of endogenous clustered DNA damage showed similar levels of DSB and non-DSB clusters in the genomically stable clones and the parental cell line. However, each of the four unstable clones studied had altered levels of particular types of damage clusters that could be distinguished from the controls. This observation suggests that varied alterations in DNA damage repair may be related to the perpetuation of the genomic instability observed in each of these clones. However, other mechanisms such as compaction of the DNA or an altered level of production of single lesions should also be considered in future studies.

2. Experimental Methods

2.1. Cell culture, cell cycle determination and analysis of chromosome stability

The human-Chinese hamster ovary (CHO) hybrid cell line, GM10115, contains a single copy of human chromosome 4 in a background of 20-24 hamster chromosomes (Human Genetic Mutant Cell Repository, Camden, NJ). The GM10115 cell line and the two stable (114, 118) and four unstable clones (CS9, LS12, 115 and Fe5.0-8) were maintained in high glucose Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and 0.2 mM L-proline at 34° C with 5% CO₂ [1, 26]. Cell cultures were routinely screened to exclude the presence of mycoplasma (Bionique Testing Laboratories, Inc., Saranac Lake, NY).

The clones were characterized by cytogenetic analysis of metaphase chromosomes using fluorescence in situ hybridization (FISH) for human chromosome 4. Details of the FISH procedure have been described previously [33]. Briefly, after exposure to colcemid mitotic cells were collected by mitotic shake-off,
fixed in methanol:acetic acid (3:1 v/v) and then dropped onto precleaned glass microscope slides and allowed to air dry. Human chromosome 4 was visualized using a biotin labeled pBluescript vector-based library of human chromosome 4-specific DNA sequences (pBS4) and subsequent hybridization protocols were performed as described previously using fluorescein-conjugated avidin and an anti-avidin antibody [33]. Chromosomes were counterstained with propidium iodide.

Metaphase chromosomes were analyzed by means of a Zeiss Axioskop microscope equipped with a dual-band pass FITC/Texas Red filter set. In this system, the hamster chromosomes appear red (propidium iodide emission), and the human chromosome 4 that hybridizes with the biotinylated probe appears yellow/green (fluorescein emission).

A stable clone was defined as one that contained a homogenous metaphase population with a unique rearrangement (translocation) or no rearrangement of the human chromosome with hamster chromosomes. An unstable clone was defined as one that contained three or more metaphase subpopulations with different rearrangements of the human chromosome. Each of these rearranged subpopulations constituted ≥ 5% of the 200 metaphases scored.

Cell cycle profiles of 90% confluent cell cultures were determined by flow cytometry. In brief, cells were harvested by trypsinization and centrifuged at 500 × g for 5 minutes. Cell pellets were resuspended in PBS and then fixed by the slow addition of 10 volumes of cold 70% ethanol while mixing. After 24 hours at 4°C, samples were pelleted by centrifugation, resuspended in a solution of PI (propidium iodide)/RNase (5 µg/ml PI, 200 µg/ml RNase, BD Pharmedgen, San Diego, CA), and incubated in the dark at room temperature for 15 minutes. 50,000 cells per sample
were then acquired by flow cytometry using a Becton Dickinson (San Diego, CA) FACS Calibur instrument to obtain a quantitative measurement of their DNA content. Cell cycle profiles were subsequently determined using Modfit software.

2.2. DNA isolation in agarose plugs

Cells from 90% confluent cultures were trypsinized, resuspended in medium at 2 × 10⁶ cells/ml in small vials and then stored at −80°C. DNA isolation in low melting-point agarose blocks was performed as described in [12] with minor modifications. In brief, each vial of frozen cells was rapidly thawed, 0.5 M EDTA (pH 8.0) was added to a final concentration of 100 mM and after mixing with an equal volume of 2% agarose (InCert, Lonza, Allendale, NJ) in TE (10 mM Tris–Cl, pH 7.5, 1 mM EDTA), the cell suspension was pipetted using wide mouth tips into plug molds which were placed at 4°C for 15 min to solidify. Agarose plugs placed in tubes were treated with lysis solution containing L-buffer (20 mM NaCl, 0.1 M EDTA, 10 mM Tris–Cl, pH 8.3), 1 mg/ml of proteinase K (Roche Molecular Biochemicals, Indianapolis, IN) and 1% n-lauroylsarcosine. Samples were incubated at 37°C for 3 days with daily changes of lysis solution. All solutions used during DNA isolation (except those containing detergents or enzymes) were argon-bubbled for 15 to 20 minutes immediately before use and tubes were gassed with argon before capping to prevent formation of additional oxidative DNA lesions [27]. Digested agarose plugs were rinsed several times with L-buffer to remove detergent and then treated twice for 30 minutes with L-buffer containing 40 μg/ml phenylmethylsulfonyl fluoride (PMSF). Samples were washed several times with ice-cold TE to remove PMSF and stored in TE at 4°C for a maximum of 2 days until further processing.
2.3. Endogenous DNA damage analysis

For clustered DNA damage determinations DNA embedded in agarose plugs from the cell lines was digested with NotI as previously described [28]. Briefly, agarose plugs containing 1300 ng of DNA per plug were treated overnight at 37°C with 5 Units/plug of NotI enzyme (New England Biolabs, USA) in NotI buffer (10 mM Tris, 10 mM MgCl₂, 150 mM NaCl) containing 1 mM dithiothreitol (DTT) and 100 µg/ml acetylated bovine serum albumin (BSA, Invitrogen, Carlsbad, CA). After treatment plugs were washed with TE and stored at 4°C.

Non-double strand break (non-DSB) clustered DNA damage was assayed following established procedures [29, 30, 31]. DNA glycosylases Fpg (E. coli Formamidopyrimidine [fapy]-DNA glycosylase) or Nth (E. coli Endonuclease III) were used to detect oxidized purine or oxidized pyrimidine clusters. Nfo protein (E. coli Endonuclease IV) was used to assess abasic clusters. All proteins were purified and tested for non-specific cleavage in the former B.M. Sutherland lab as reported previously [12]. For lesion-specific enzyme treatment, each DNA plug was divided and each half was transferred to 70 mM Hepes/KOH (pH 7.6), 100 mM KCl, 1 mM EDTA (pH 7.6), and then to the same buffer with 1 mM DTT and 10 µg/ml BSA at 4°C. One half plug was incubated at 37°C for 20 h with buffer alone while the other half was treated with sufficient quantities of enzyme to cleave at all cluster sites. For each enzyme treatment one half plug 650 ng of DNA, 120 ng Fpg protein, 150 ng Nth protein or 80 ng endonuclease IV was used. These amounts were derived based on titration of the activity for each enzyme assayed using the parental GM10115 cell line (Figure 1). After glycosylase/endonuclease treatment was complete, any traces of enzyme were removed by incubation with lysis solution at 37°C overnight. Plugs were then rinsed and stored in TE at 4°C. Companion plug samples for
determination of DSBs were kept in TE at 4° C. To measure clustered DNA damage, samples along with DNA molecular length standards (*Schizosaccharomyces pombe* (3.5–5.7 Mbp), *Saccharomyces cerevisiae* (0.225–2.2 Mbp) (BioRad) and Hind III digest of bacteriophage λ (0.5–48.5 kbp) were electrophoresed on 0.8% SeaKem Gold agarose gels (Lonza) in 0.5X TBE (1X TBE: 90 mM tris borate, 2 mM EDTA, pH 7.8). Neutral pulsed-field electrophoresis was performed using a CHEF apparatus (Gene Navigator, GE Healthcare, Piscataway, NJ) at 8° C in 0.5X TBE at 5 V/cm for 8 h followed by 3 V/cm for 30 h, applying a linearly ramping pulsing regime from 0 s to 1200 s. Gels were stained with 1 µg/ml ethidium bromide in double distilled water for 30 min, destained for 4 h and then a quantitative electronic image was obtained. A DNA dispersion curve was determined using the described DNA length standards and the average DNA length (Ln) distribution was computed for each sample. From these Ln values, the frequencies of DSBs, oxidized base or abasic clusters were calculated [30, 32]. In brief, the frequency of DSBs (ΦDSB) for each clone was calculated and normalized to the parental cell line as in the equation

ΦDSB = 1/Ln (clone) – 1/Ln (parental strain) where 1/Ln is the reciprocal of the average length distribution. The frequencies of oxypurine, oxypyrimidine and abasic clusters (ΦC), were similarly calculated from the following equation ΦC = 1/Ln(+) - 1/Ln(-) where 1/Ln(+) and 1/Ln(-) are reciprocals of number average lengths of sample pairs with and without enzyme, where the enzymes are Fpg, Nth and Nfo enzymes, respectively.

2.4. Statistical analysis

Data from four independent determinations are given in each figure, and standard errors of the means were plotted using SigmaPlot. Statistical analysis was
performed using Student’s two-tailed $t$-tests. $P$ values of $\leq 0.05$ were considered statistically significant and indicated by asterisks in the figures.

3. Results

To test the hypothesis that an altered level of endogenous clustered DNA damage is related to persistent genomic instability we have determined the frequencies of clustered DNA damage, including DNA DSBs and non-DSB clustered lesions, using genomically stable and unstable cell lines previously derived from the irradiated human–hamster hybrid GM10115 cell line [1, 26].

3.1 Analysis of chromosomal stability and cell cycle determinations

These stable and unstable cell lines were established and originally characterized by Limoli and colleagues following irradiations that took place in 1997 in the case of the CS9, LS12, 115, 114, and 118 clones [1], and in 2000 in the case of the Fe5.0-8 clone [26]. Over the past 15-18 years, these cells have been propagated and used in numerous studies. In the present study each clone was re-characterized for genomic instability by cytogenetic analysis of metaphase chromosomes (Table 1). The clones that had originally been characterized as genomically unstable still meet that criteria. The number of subpopulations for the unstable clones has decreased since they were first characterized more than a decade ago. Based on cytogenetic comparison of the parental cell line and the stable clones to the unstable clones, the reduction in the number of different
subpopulations is most likely attributed to loss of human chromosome 4 content over time in the course of chromosomal rearrangements.

**Table 1.** Cytogenetic re-classification of cell lines.

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Radiation</th>
<th>Classification</th>
<th>No. Subpopulations&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Aberrant Metaphases&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Metaphases Scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM10115</td>
<td>None</td>
<td>Parental</td>
<td>1</td>
<td>0%</td>
<td>200</td>
</tr>
<tr>
<td>114 [1]</td>
<td>10 Gy X-Rays</td>
<td>Stable</td>
<td>1</td>
<td>100%</td>
<td>200</td>
</tr>
<tr>
<td>118 [1]</td>
<td>10 Gy X-Rays</td>
<td>Stable</td>
<td>1</td>
<td>0%</td>
<td>200</td>
</tr>
<tr>
<td>CS9 [1]</td>
<td>10 Gy X-Rays</td>
<td>Unstable</td>
<td>4</td>
<td>100%</td>
<td>200</td>
</tr>
<tr>
<td>LS12 [1]</td>
<td>10 Gy X-Rays</td>
<td>Unstable</td>
<td>5</td>
<td>100%</td>
<td>207</td>
</tr>
<tr>
<td>115 [1]</td>
<td>10 Gy X-Rays</td>
<td>Unstable</td>
<td>4</td>
<td>100%</td>
<td>206</td>
</tr>
<tr>
<td>Fe5.0-8 [26]</td>
<td>5 Gy Fe Ions</td>
<td>Unstable</td>
<td>3</td>
<td>95%</td>
<td>200</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of different abnormal metaphase subpopulations showing distinct types of chromosome alterations.

<sup>b</sup> Percentage of metaphases scored showing chromosome aberrations.

Recent determinations of abasic site density in DNA by fiber analysis or standard slot blot assays have shown an increased frequency of abasic clusters in areas where DNA replication is in progress [34]. Therefore, a low percentage of cycling cells in the analyzed culture is important to minimize the background level of typical abasic site clusters and DSBs due to DNA replication. For this reason, the parental GM10115 cell line, stable clones 114, 118 and cytogenetically unstable clones LS12, CS9, 115, Fe 5.0-8 were cultured under optimal conditions and grown to more than 90% confluence before harvesting. Cell cycle distributions of cells for the parental cell line and each clone were assessed by flow cytometry analysis. Under these culture conditions, most of the cell were at G1 in the parental cell line and with the exception of the 115 unstable clone, the percentage of cells in S and G1 were similar among all clones (Figure 2).
3.2 Frequencies of different types of endogenous non-DSB clustered DNA damage

Different types of endogenous non-DSB clustered DNA damage are present in normal cells as well as in tumor cells [11, 12, 35, 36, 37, 38, 39]. These endogenous clusters include oxidized bases and abasic site clusters that can be detected using lesion-specific enzymes. The enzymes recognize and cleave DNA containing these types of lesions generating DNA DSBs that can be quantified by pulsed field gel electrophoresis and number average length analysis. It should be noted that only clustered lesions with opposing DNA damage can be detected by this method. In the present work, the levels of oxidized base clusters were assayed using the lesion-specific enzymes Fpg glycosylase that recognizes and cleaves DNA containing oxypurines and Nth glycosylase that recognizes oxypyrimidines. Although not statistically significant, in all the experimental determinations the frequency of endogenous Fpg-oxypurine clusters, expressed as the number of clusters per Gbp, was slightly increased in the unstable clone LS12 in comparison with the parental cell line and the other stable and unstable clones studied (Figure 3A).

The frequencies of endogenous Nth-oxypyrimidine clusters are presented in Figure 3B. As previously reported in human and other mammalian cell lines [12, 40], the observed levels of oxypyrimidine clusters were lower than the oxypurine clusters in our cells. The frequencies of endogenous oxypyrimidine clusters were similar in the parental cell line, the stable clones 114 and 118 and in the unstable clones CS9 and Fe5.0-8. Surprisingly, a decreased level of Nth-oxypyrimidines clusters was observed in the unstable clones 115 and LS12. It should be noted that while the decrease observed repeatedly in the experiments for the LS12 cells was within experimental error, the decreased level of Nth-recognized clusters observed in 115 cells was statistically significant.
In different mammalian cell lines DNA abasic site clusters are also observed as endogenous damage, although at lower levels than oxidized bases [11, 35]. Base loss can occur spontaneously as a consequence of endogenous oxidative damage or arise as repair intermediates of base damage processing [41, 42, 43]. Since some preparations of the *E. coli* Nfo enzyme can incise abasic sites and oxidized pyrimidines, we used a homogeneous Nfo protein tested to be devoid of detectable AP-lyase and DNA glycosylase activities [12]. Figure 3C shows that only the genomically unstable clones 115 and LS12 presented significantly altered levels of Nfo-clusters. The abasic site cluster frequency was increased in LS12 clone and reduced in 115 as compared with the parental cell line.

### 3.3 Presence of endogenous DSBs in confluent cell cultures of genomically stable and unstable clones

DSBs can be produced by oxidative DNA damage caused by ROS generated during normal metabolism, arise when single strand breaks are in close proximity during the processing of non-DSB clustered DNA damage, or following replication of DNA containing ROS-induced lesions. In non-dividing cells the number of endogenous DSBs is expected to be low as observed in G0 human fibroblasts [44, 45]. Figure 4 shows the frequencies of DSBs in each cell line normalized to the DSB frequency in the parental cell line. In the stable clones 114 and 118, and the unstable clone Fe5.0-8 the levels of DSBs remained low, with no statistically significant difference as compared to the parental cell line. However, the three unstable clones CS9, LS12 and 115 exhibited a significant increase in the number of endogenous DSBs. Interestingly, although CS9 did not show increased non-DSB frequencies, it had the highest endogenous DSB frequency.
3.4 Spectrum and total levels of endogenous clustered DNA damage

The spectrum of clustered DNA damage constitutes an important factor related to structural complexity that may pose a significant challenge to the rate and effectiveness of cellular DNA repair systems and replication [46, 47]. Therefore, we questioned whether the variable frequencies of the different classes of non-DSB clustered DNA damage observed in each cell line represent a modification of the total number of non-DSB endogenous clusters or a different spectrum of clusters. The total frequencies of non-DSB clusters were calculated for each clone as the sum of the frequencies (and the corresponding statistical errors) of the assessed classes of non-DSB damage and are presented in Figure 5A. Although the unstable clone LS12 showed a slight increase in the total frequency of non-DSB clusters, only the 115 clone presented a statistically significant change. In this unstable clone the level of total endogenous non-DSB clustered damage was lower compared with the parental cell line. To assess the relative contribution of DSBs and non-DSB clusters to the total clustered DNA damage, the total frequency of damage was calculated. While the unstable clones CS9 and LS12 had higher frequencies of endogenous clusters these differences were not statistically significant (Figure 5B). All other clones showed frequencies of endogenous clusters similar to the parental cell line.

4. Discussion

Low levels of clustered DNA damage are generated by endogenous ROS during normal aerobic metabolism and/or by the persistence of lesions due to discrete leaks in the repair processing of these ROS-induced and/or replication damage. Therefore, the levels of endogenous clustered DNA damage may be
affected by cellular genotype and/or the oxidative state of the cells through interrelated mechanisms that may impact the level and rate of production of damage clusters and their removal. In fact, while low frequencies of endogenous clusters are detected in normal human cells and tissues, an increase in oxidized base clusters is observed in radiation-sensitive hematopoietic cell lines presenting low glycosylase levels [11, 12, 35, 36]. In addition, environmental changes that may induce an increase in cellular oxidative stress, such as that produced by tobacco smoke [48], have been shown to increase the levels of endogenous clustered DNA damage [12]. Furthermore, high levels of oxidative clustered DNA damage have been measured in cells from malignant tumors [37] where genomic changes and increased oxidative stress may coexist.

The nucleus and mitochondria modulate each other’s activities through bidirectional signaling cascades [24, 25, 49] and alterations in these signaling networks are complexly interrelated in carcinogenesis. Based on the hypothesis that modified levels of endogenous clustered DNA damage is related to the persistence of radiation induced genomic instability we examined the frequencies of different types of clustered DNA damage in cells exhibiting radiation induced genomic instability and in genomically stable clones whose progenitors had also been irradiated. The analysis of endogenous clustered DNA damage shows that while the two genomically stable clones have levels of DSB and non-DSB clusters similar to the parental cell line, three of the four unstable clones had variable levels and spectra of clusters that could be distinguished from the parental cell line. It is interesting that the unstable clone that was exposed to high linear energy transfer (LET) iron ion radiation, Fe5.0-8 did not have markedly elevated levels of DSB or non-DSB clusters. While clearly not conclusive since only one clone derived from Fe
particle irradiation was evaluated, these data suggest that follow up studies might
explore possible differences in radiation responses and the quality of DNA damage
in the genomically unstable progeny cell based on the quality of the radiation to
which the surviving cell had been exposed.

Endogenous oxidized base clusters were detected in all cell lines. While the
frequencies of oxypurines were higher than the oxypyrimidine clusters, the level of
Fpg oxypurine clusters was similar in all of the cell lines (Figure 3). Although the
unstable clones CS9 and LS12 have been previously shown to exhibit mitochondrial
dysfunction and increased oxidative stress [4, 19, 50], the levels of oxypurine or
oxypyrimidine clusters were not significantly elevated when compared to the parental
GM10115 cell line or the other clones tested. Interestingly, the frequencies of Nth-
recognized oxypyrimidine clusters were decreased in the unstable clone 115. It is
possible that these clones have constitutively upregulated DNA repair signaling
and/or there is a decrease in endogenous production of oxidized clusters. However,
the latter explanation is less likely in the case of clone 115 since oxidative damage
induced endogenous oxypurine clusters were produced at similar levels in all cell
lines. Increased sensitivity to bleomycin, a radiomimetic antitumor antibiotic that
induces damage more similar to densely ionizing high LET radiations than to
sparsely ionizing low LET radiations [51, 52], was shown in the radiosensitive CHO
cells transfected with *E. coli NTH* gene [53]. Additionally, overproduction of Nth
glycosylase resulted in significantly increased DSB formation after irradiation using
human lymphoblastoid TK6 cells [54].

The evaluation of abasic sites showed that the unstable clone LS12
presented the highest levels of this damage of all the cell lines tested (Figure 3). This
increase is in accordance with the clone’s cellular phenotype that includes increased
intracellular ROS and dysfunctional mitochondria [4], although no significant increase in oxidized bases clusters was detected. However, impaired function of BER involving the apurinic/apyrimidinic endonuclease cannot be discounted. Interestingly, the unstable clone 115 showed lower frequency of abasic clusters (Figure 3) while presenting a high fraction of cells in S phase (Figure 2) as compared to the other cell lines. However, this clone showed a high frequency of DSBs (Figure 4) that could be related in part to this high fraction of cells in S phase. This lower total level of endogenous non-DSB clusters (Figure 5A) and increased frequency of DSBs as compared to the other cell lines suggest an altered function of the BER pathway. Notwithstanding, it has been shown that overexpression of some enzymes involved in the BER pathway may be detrimental to a cell since an imbalance in physiological enzyme levels may lead to genomic instability [55].

DSBs of different complexity may be induced by endogenous oxidative stress. Also, clustered DNA damage containing oxidized bases and/or abasic sites processed by BER processes may give rise to potentially repair-resistant DSBs [45, 54]. The impact of damage complexity on DSB repair is evident since recombinational and other repair pathways including BER may be needed to process these types of damage [44]. We observed a significant increase in the level of DSBs in the unstable clones LS12, CS9 and 115 (Figure 4). In the LS12 clone the higher level of DSBs could be related to the increased level of abasic site clusters, possibly due to incomplete processing by BER. Finally, in the case of the CS9 clone, in the absence of elevated levels of non-DSB clusters (Figure 5A), the increase in DSB frequency could be due to defects in DSB processing. In fact, mouse cells deficient in non-homologous end joining repair exhibit elevated genetic instability suggesting the importance of this repair pathway in processing endogenous DSBs [44].
Interestingly, while not statistically significant the total frequency of endogenous clustered DNA damage was elevated in the CS9 and LS12 unstable clones (Figure 5B). Surprisingly, the unstable clone 115 also exhibited a similar level of total endogenous clustered DNA damage compared with the parental cell line GM10115. Given that the unstable clones CS9, LS12 and 115 exhibited different spectra of clustered DNA damage, it is not surprising that the clones would also have changes in efficiencies of DNA repair pathways. However, other mechanisms such as compaction of the DNA or an altered level of production of single lesions should be considered. Further cellular and molecular studies are necessary to test these hypotheses regarding the DNA repair capabilities of these genomically unstable clones. Nevertheless, the DNA damage spectra observed in these cells clearly reinforce the message that radiation induced genomic instability is a multifactorial phenomenon.

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Conflict of Interest

The authors declare no conflict of interest.

References


28. Das, B.; Bennett, P. V.; Cutter, N. C.; Sutherland, J. C.; Sutherland, B. M., Melatonin protects human cells from clustered DNA damages, killing and acquisition of soft agar growth induced by X-rays or 970 MeV/n Fe ions. *International Journal of Radiation Biology* **2011**, **87**, (6), 545-555.


Figure Legends

Figure 1. Titration of GM10115 cell line DNA with A) Fpg (circles), B) Nth (triangles) or C) Nfo (square) enzymes to assess the amount of Fpg, Nth and Nfo proteins required for optimal cleavage. DNA in agarose plugs was treated with increasing amounts of Fpg, Nth or Nfo proteins under appropriate reaction conditions (see Experimental Methods). Each symbol corresponds to the averages ± SE of two independent replicate experiments.

Figure 2. Cell cycle distributions of GM10115 cells, stable clones 114 and 118, and unstable clones CS9, LS12, 115 and Fe5.0-8 as verified by flow cytometry analysis.

Figure 3. Endogenous non-DSB clustered DNA damage in stable and unstable clones. A) Endogenous Fpg-recognized oxypurine clusters, B) endogenous Nth-recognized oxypyrimidine clusters, and C) endogenous Nfo-abasic clusters. Parental cell line GM10115 (light gray), derived stable clones 114 and 118 (gray) and unstable clones CS9, LS12, 115 and Fe5.0-8 (dark gray). Each column represents the mean frequency of Fpg clusters/Gbp ± SE of 4 independent replicate experiments.

Figure 4. Endogenous DNA double strand breaks in the parental cell line GM10115 (light gray), derived stable clones 114 and 118 (gray) and unstable clones CS9, LS12, 115 and Fe5.0-8 (dark gray). Each column represents the mean frequency of DSBs/Gbp ± SE of 4 independent replicate experiments. The frequency of DSBs for each cell clone was calculated normalized to the parental cell line, *P < 0.05.
Figure 5. A) Total endogenous levels of non-DSB clustered DNA damage and B) Total endogenous levels of clustered DNA damages in the parental cell line GM10115 (light gray), derived stable clones 114 and 118 (gray) and unstable clones CS9, LS12, 115 and Fe5.0-8 (dark gray). Each column represents the mean frequency of the sum of DSB plus non-DSBs clusters/Gbp ± SE.
Fig. 2

![Bar chart showing cell line distribution](chart.png)
Fig. 4

The figure shows a bar graph with the x-axis labeled "Cell Line" and the y-axis labeled "DSB/Gap^1". The cell lines are GM, 114, 118, CS9, LS12, 115, and Fe5.0-8. The graph includes error bars and asterisks indicating statistical significance.