Formation and repair of clustered damaged DNA sites in high LET irradiated cells

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Abstract

Purpose: Radiation with high linear energy transfer (LET) produces clustering of DNA double-strand breaks (DSB) as well as non-DSB lesions. Heat-labile sites (HLS) are non-DSB lesions in irradiated cells that may convert into DSB at elevated temperature during preparation of naked DNA for electrophoretic assays and here we studied the initial formation and repair of these clustered damaged sites after irradiation with high LET ions.

Materials and methods: Induction and repair of DSB were studied in normal human skin fibroblast (GM5758) after irradiation with accelerated carbon and nitrogen ions at an LET of 125 eV/nm. DNA fragmentation was analyzed by pulsed-field gel electrophoresis (PFGE) and by varying the lysis condition we could differentiate between prompt DSB and heat-released DSB.

Results: Before repair (t = 0 h), the 125 eV/nm ions produced a significant fraction of heat-released DSB, which appeared clustered on DNA fragments with sizes of 1 Mbp or less. These heat-released DSB increased the total number of DSB by 30–40%. This increase is similar to what has been found in low-LET irradiated cells, suggesting that the relative biological effectiveness (RBE) for DSB induction will not be largely affected by the lysis temperature. After 1–2 hours repair, a large fraction of DSB was still unrejoined but there was essentially no heat-released DSB present.

Conclusions: These results suggest that high LET radiation, as low LET gamma radiation, induces a significant fraction of heat-labile sites which can be converted into DSB, and these heat-released DSB may affect both induction yields and estimates of repair.

Keywords: Clustered damage, DNA damage, DNA DSB repair, DNA repair, double-strand breaks, High LET

Introduction

Ionizing radiation with high linear energy transfer (LET) produces clustered damaged sites within the DNA. The resulting DNA damage is believed to be more complex than that induced by low LET radiation and leads to more severe biological effects (Goodhead 1994). In addition to DNA double-strand breaks (DSB), ionizing radiation also induces single-strand breaks (SSB), modifications of the DNA bases and deoxyriboses, DNA-DNA and DNA-protein cross-links, as well as the occurrence of alkali- and heat-labile sites (Ward 1985). Although the DSB is considered to be the most critical lesion relating to cell survival, chromosomal aberrations or other late cellular effects, non-DSB clusters, e.g., combination of two or more oxidized base, strand break or other DNA lesion that do not form a DSB (reviewed by Georgakilas et al. 2013), some of which may be transformed into DSB, add to the damage complexity of DSB and thereby affect their repair. Further, DNA lesions may also be generated during cellular processing of a primary damage, or throughout the preparation of DNA, and these additional DSB cannot be distinguished from the prompt DSB formed directly by ionizing radiation.

Besides inducing DSB with a high degree of complexity, radiation with high LET, such as α-particles and accelerated ions, has been shown to produce correlated, non-randomly distributed DSB in intact cells (Löbrich et al. 1996, Rydberg 1996, Newman et al. 1997, Höglund et al. 2000). These studies separated the fragmented DNA by pulsed-field gel electrophoresis (PFGE) over a wide size range and found an excess of DNA-fragments less than 1 Mbp after high-LET radiation, which cannot be detected by standard PFGE assays. Low-LET radiation produced no or only minor deviations from random distribution. Thus, the relative biological effectiveness (RBE) for these small- and intermediate-size fragments is relatively high compared to DSB analyzed without taking fragment distributions into account.

One example of a non-DSB clustered lesion is the heat-labile site (HLS), which is revealed as strand breaks after thermic treatment of DNA from irradiated plasmid DNA or mammalian cells (Von Sonntag and Schulte-Frohlinde 1978, Jones et al. 1994, Henle et al. 1995). A double-stranded HLS or a SSB opposite a HLS could be converted into a DSB
during the cell lysis step at elevated temperatures in the preparation of naked genomic DNA for analysis by constant-field or pulsed-field gel electrophoresis (PFGE). The conversion of heat-labile sites into DSB increases the initial number of DSB (t = 0) by 35–40% or more in gamma-irradiated human cells (Rydberg 2000, Stenerlöw et al. 2003) and it is evident that the post-irradiation temperature will have a significant effect on the estimated DSB yields. The biological impact of heat-labile sites in irradiated cells is still not known but we suggested that conversion of HLS into DSB is merely an artifact in the analysis, which mainly take place when naked DNA is exposed to elevated temperatures (Stenerlöw et al. 2003, Karlsson et al. 2008). Further, the repair of HSL was independent of DNA-dependent protein kinase (DNA-PK), suggesting that removal of HLS is not coupled to major DSB repair pathways (Gulston et al. 2004, Karlsson et al. 2008). In contrast to these observations, recent results indicate that heat-labile sites may develop into DSB also in vivo (Singh et al. 2011). Irrespective of their biological significance, detection of HLS could provide important information on clusters of non-DSB lesions and their repair.

So far there is limited information about the contribution of HLS to the DSB measurements in cells irradiated with high LET ions but recent data suggest that the fraction of HLS is reduced when the LET is increased (Singh et al. 2013). Since high-LET radiation produces an excess of DNA-fragments less than 1 Mbp after high-LET radiation, which cannot be detected by standard PFGE assays, the aim of the present investigation was to determine the distribution of DSB and HLS in cells irradiated with high-LET ions using DNA fragmentation analysis. At t = 0 we found that 125 eV/nm ions produced a considerable fraction of non-DSB lesions which were released into DSB upon heat treatment. These heat-converted DSB appeared non-randomly distributed and were almost exclusively increasing the number of DNA fragments with sizes smaller than 1 Mbp. Further, after 1–2 h repair incubation, these non-DSB clusters were completely removed but there was still a significant fraction of unrejoined DSB.

Materials and methods

Cells and irradiations

Low passages of normal human skin fibroblast GM5758 (Human Genetic Mutant Cell Repository, Camden, NJ, USA) were labelled with 1500–2000 Bq/ml [methyl-14C]thymidine (Perkin Elmer, Waltham, MA, USA) in Eagle minimal essential medium (MEM), supplemented with 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin, 2 × concentration of MEM vitamins and both non-essential and essential amino acids (all from Biochrom Kg, Berlin, Germany). Cells were grown in 35 mm Petri dishes and irradiated on ice at a dose-rate of 10–30 Gy/min at an average LET of 125 eV/nm. For PFGE studies (see below) the radiation dose was 80 Gy and 100 Gy for the carbon ions and nitrogen ions, respectively. Cells for repair were irradiated as monolayers (Stenerlöw et al. 2000), whereas for the t = 0 h time-point (‘induction’) cells were irradiated in an agarose plug (see below). The cell culture was confluent at the time of irradiation.

Ion beam dosimetry

Ion irradiations were carried out at the Biomedical Unit at The Svedberg Laboratory, Uppsala University, Sweden. Ions from an external ion source were accelerated in the Gustaf Werner synchrocyclotron to an energy of approximately 40 MeV/u and the beam was scattered by an 80 μm gold foil placed 3.3 m before the samples. A vacuum tube was placed between the scattering foil and the samples to reduce the energy loss. The dose-distribution at the cell position was homogenous within ±5% in a diameter of 35 mm. Absolute dosimetry and lateral dose-distributions were measured by a calibrated ionization chamber with a 2.76 mg/cm² polystyrene window (Markus type 23343, PTW, Freiburg, Germany) placed on an X-Y-scanner. Irradiation doses to cells were monitored by a plane-parallel transmission ion-chamber in front of the sample. LET (stopping power) at cell positions was determined from theoretical energy-LET-distributions (Ziegler and Manoyan 1988). The depth-dose distribution of the carbon ion beam is shown in Figure 1. Note the resolution of the measurement in the inserted figure (data points are separated by typically 5 μm, less than one cell diameter). Complementary dosimetry was performed by using nuclear track detectors (CR-39, Pershore Moulding Ltd, Pershore, UK), see Supplementary information for details (available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1068463) and there was a 1:1 correlation between the tracks and 53BP1 foci (Supplementary Figure 1 available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1068463). Nitrogen ion irradiation was performed as described previously (Höglund et al. 2000).

Pulsed-field gel electrophoresis (PFGE)

Cells for PFGE analysis were embedded in 20 μl (1 × 4 × 5 mm) agarose plugs (0.6% w/v, InCert, Cambrex Bio Science Rockland, Rockland, ME, USA) at a concentration of 2–2.5 × 10⁶ cells per ml as previously described (Stenerlöw et al. 2003). The stated repair time span from the addition of fresh 37°C medium to the incubation at 4°C. Briefly, following irradiation cells were lysed at 50°C overnight in ESP buffer containing 0.5 M EDTA, pH 8.0 2% N-lauroylsarcosine, and 1 mg/ml protease K (Roche Diagnostics, Mannheim, Germany), or using a cold two-step protocol to avoid the release of heat-labile sites (Stenerlöw et al. 2003). In this protocol the plugs were kept in cold ESP (0°C) following irradiation. After >20 h the ESP buffer was removed and replaced with HS-buffer and incubated overnight at 0°C (HS: High Salt; 1.85 M NaCl, 0.15 M KCl, 5 mM MgCl₂, 2 mM EDTA, 4 mM Tris, 0.5% Triton X-100, pH 7.5). The naked DNA was separated by PFGE with two different protocols optimized for separation of DNA fragments in the size range 5 kbp–1.5 Mbp and 1–6 Mbp as described previously (Höglund et al. 2000). Gels were sliced using DNA the size markers Schizosaccharomyces pombe (Bio-Rad Laboratories, Hercules, CA,
USA), *Saccharomyces cerevisiae* and Lambda DNA-PFGE marker (both from New England Biolabs, Beverly, MA, USA) followed by measurement of DNA-incorporated $^{14}$C in each gel segment by liquid scintillation.

**DSB quantification**

The total number of DSB was calculated by fragment analysis, taking the DNA fragment distribution in the interval 48 kbp–5.7 Mbp, into account for each time-point as previously described (Högglund et al. 2000). Briefly, the number of DNA fragments in each size zone is proportional to the amount of radioactivity in the corresponding gel segment. By dividing the fraction of DNA, $F_i$, in the gel segment $i$, with the mean fragment size in the segment, $M_i$, the number of fragments, $n$, can be calculated and the total number of fragments is then given by the sum of yields for all size-intervals.

**Results**

Initial (t = 0 h) DNA fragment distribution was analyzed after irradiation with accelerated ions (LET = 125 eV/nm). Cells were irradiated and naked DNA was prepared by lysis at two different temperatures (‘cold,’ 0°C and ‘warm,’ 50°C, see Materials and methods for details) and the normalized fraction of DNA was plotted as a function of DNA fragment size. For both the carbon ions (Figure 2A) and nitrogen ions (Figure 2C) the warm lysis resulted in a larger fraction of released DNA. Further, the non-DSB lesions which are converted into DSB upon heat treatment appear clustered on DNA fragments with sizes of 1 Mbp or less. This is clearly shown when the differences between the fractions of DNA are plotted in Figure 2B and 2D.

To further evaluate the effect of different lysis on the DNA fragment distributions we plotted the fraction of DNA as a function of dose for four different cut-off sizes. The data were obtained by re-plotting results from two studies using the same experimental setup but different lysis protocols, i.e., warm lysis (Högglund et al. 2000) and cold lysis (Radulescu et al. 2004). For the fraction of DNA < 5.7 Mbp, comparable to standard PFGE assays, there was no significant difference between the two protocols but as the exclusion size decreases, the warm lysis was more effective in releasing DNA (Figure 3). Thus it is evident that the heat-released DSB are mainly clustered on small DNA fragments, less than 1 Mbp in size (Figure 3).

To study how the additional heat-released DNA fragments (< 1 Mbp) changed over time, we compared the DNA fragment distributions following repair after irradiation with ions (Figure 4). The DNA fragments analyzed after both treatments were relatively rapidly rejoined and already after 0.4 h there was a 2- to 5-fold decrease in the fractions of small DNA fragments after irradiation with carbon ions (Figure 4A and B), but the rejoining appeared faster in the heat-treated samples. This was also evident in cells irradiated with nitrogen ions where a large fraction of the small DNA fragments were rejoined within 0.1 h (Figure 4C).

When the total number of DSB over all fragment-sizes (less than 5.7 Mbp) was calculated, the initial yields at t = 0 was similar for both C and N ions, resulting in about 45 prompt (0°C) DSB/cell/Gy and 15 additional heat-released DSB/cell/Gy (Table 1). During the repair incubation, there was no apparent difference in repair for cells irradiated with C or N ions (Figure 5). The initial difference in yields for the two protocols decreased, and 1–2 h after irradiation, there was no evident difference in the number DSB measured by the two protocols (0 and 50°C).
are non-randomly distributed (Radulescu et al. 2004, Claesson et al. 2007). Thus, after high-LET irradiation both DSB and non-DSB lesions seem clustered within Mbp-sized chromatin structures (Radulescu et al. 2004) or larger nuclear domains (Costes et al. 2006, Fakir et al. 2006).

These data contrast recent results for cells irradiated with neutrons (Singh et al. 2012) and Fe ions (Singh et al. 2013), where HLS were absent, or largely reduced, compared to HLS formation seen after gamma irradiation. Although differences in ionization patterns and formation of clustered damaged sites may lead to different HLS:DSB ratios for high- and low-LET radiation, we believe the main discrepancy can be explained by the methodology used to detect HLS and DSB. Since heat-released DSB seems clustered on small DNA fragments after high-LET irradiation, these fragments would escape detection in standard PFGE assays, measuring mainly large DNA fragments. This is clearly illustrated in Figure 3; when using a large cut-off size as in the standard PFGE analysis (Figure 3A, DNA < 5.7 Mbp), there was no difference in the DSB yields measured after warm (50°C) or cold (0°C) lysis. This pinpoints the importance to use DNA

Figure 2. Initial DNA fragmentation (t = 0) as a function of DNA fragment size after irradiation of human fibroblast cells with carbon ions (80 Gy) or nitrogen ions (100 Gy) both with a LET of 125 eV/nm. Cells were irradiated and naked DNA was prepared by lysis with two different protocols (0 and 50°C, see Materials and methods for details). The distributions of double-stranded DNA fragments are shown for the two lysis protocols after irradiation with carbon ions (A) and nitrogen ions (C). The fraction of heat-released DNA fragments at t = 0, obtained by subtraction of the fraction of DNA for the 0°C lysis from the fraction of DNA for the 50°C lysis, after irradiation with carbon ions (B) and nitrogen ions (D). Mean and SEM from three experiments with 1–2 gel samples per experiment.

Discussion

Standard PFGE assays typically separate DNA of Mbp-sizes and the quantification of DSB is based on a random breakage model (Blocher 1990), or simply as the fraction of DNA released in the gel. However, high-LET radiation produces an excess of small- and intermediate-sized DNA and standard PFGE, or other assay that monitors production of essentially large DNA fragments, will most likely underestimate the yields of DSB induced such type of radiation (Prise et al. 1998). In the present study we therefore used DNA fragment analysis to evaluate DNA double-strand break induction and rejoining after ion irradiation. The non-DSB lesions, which are converted into DSB upon heat treatment, appeared clustered on DNA fragments with sizes of 1 Mbp or less (Figure 2) which was also confirmed in the analysis in Figure 3. With these three sets of experiments, including evaluation of published data, we clearly showed that high-LET radiation induces clustered damaged sites that are converted into DSB upon heat-treatment. However, it is important to note that eliminating the heat-released DSB did not change the fact that high-LET-induced DSB
fragment analysis for the evaluation of DNA damage and repair after high-LET irradiation.

We found that DSB yields in human fibroblasts after exposure to high-LET radiation increase with similar amounts when samples are heat-treated as previously have been found for the same human fibroblasts irradiated with low-LET gamma radiation (Stenerlöw et al. 2003, Karlsson et al. 2008). This suggests that the RBE for DSB induction will not be largely affected by the lysis protocols presented here, which is important for the interpretation of previous studies where PFGE data have been obtained by using warm lysis protocols. In a previous study, using cells exposed to the high-LET Auger emitter $^{125}$I, the yield increased with around 20% when heat-released DSB were included in the analysis (Elmroth and Stenerlöw 2005). However, in these experiments cells were irradiated under a high scavenging condition (10% DMSO), which reduces the indirect effect of the radiation, and possibly also the yields of lesions involved in the formation of heat-released DSB (e.g., HLS and SSB).

Because the initial number (at $t = 0$ h) of DSB was 30–40% higher in heat-treated DNA, this indicates that the rejoicing capacity could be overestimated if heat-released DSB are included in the analysis. These data are also in line with results from gamma-irradiated cells where all heat-released DSB were removed from the analysis after <1 h repair incubation (Stenerlöw et al. 2003, Gulston et al. 2004, Karlsson et al. 2008) and consequently, the fraction of DSB rejoined by a fast kinetics decreased, but without affecting the half-times of neither the fast nor slow DSB rejoining. In the present study with high-LET irradiated cells, the removal of heat-released DSB may appear slower than previously reported for low-LET irradiated cells, although there are too few data points for robust curve fitting and accurate estimate of rejoicing parameters. However, it cannot be excluded that clustering of lesions involved in the conversion into heat-released DSB (e.g., HLS, SSB) after high-LET irradiation may affect the removal of heat-released DSB from the analysis. If a cluster comprises several heat-labile sites on both strands of the DNA double helix, or on one strand with one or more SSB on the opposite strand, a heat-released DSB could be formed even if some SSB or HLS have been repaired. The ionization pattern differs between low- and high-LET irradiation but it has been suggested that OH radicals are the probable precursors to the induction of HLS in DNA irradiated in aqueous solution under scavenging conditions after both low- and high-LET irradiation (Yokoya et al. 2003). Notably, the contribution of HLS to the overall DSB yield is cell-type dependent (Singh et al. 2009) and this might affect

Figure 3. Fraction of DNA as a function of nitrogen ion (125 eV/nm) dose for human fibroblast cells. Data are plotted for different exclusion sizes of the double-stranded DNA fragments, (A) $<5700$ kbp, (B) $<930$ kbp, (C) $<680$ kbp, and (D) $<375$ kbp. Data for lysis at 50°C were obtained from (Högland et al. 2000) and data for lysis at 0°C were obtained from (Radulescu et al. 2004). Mean and SD from 4–6 experiments.
DNA damage evaluation also after high-LET irradiation (Singh et al. 2012, 2013). The exact nature of heat-labile sites generated by radiation is not known, but damage to sugar-phosphate residues are potential labile sites that could be converted into strand breaks upon heating and recent data indicates that sugar modification can be destabilized within nucleosomal DNA (Zhou and Greenberg 2012). The potential role of HLS in living cells is not known, but since HLS are partly released also at 37°C (Stenerlöw et al. 2003, Singh et al. 2011), they may have the possibility to affect both DSB formation and repair.

In summary, we found that clustered non-DSB lesions in high-LET irradiated cells can be converted into DSB upon heat treatment. The initial yield (at \( t = 0 \) h) of DSB in nor-

**Table I. Yields of prompt, total and heat-released double-strand breaks (DSB)\(^a\).**

| Type of DSB      | C ions | N ions |
|------------------|--------|--------|
| Prompt (0°C)     | 46     | 43     |
| Total (50°C)     | 63     | 57     |
| Heat-released    | 17     | 14     |

\(^a\) Yields in DSB/cell/Gy determined at a single radiation dose (80 Gy and 100 Gy for C ions and N ions, respectively) assuming \( 6 \times 10^9 \) bp per diploid cell in G0/G1 phase.
nanal human fibroblast cells increased with 30–40% due to additional heat-released DSB, which is similar to what has been seen in low-LET irradiated cells. Thus, the conversion of heat-labile sites into DSB seems relatively independent of LET and radiation-type, indicating that the RBE for DSB induction will be similar when high temperature lysis or low temperature lysis are used. The additional DSB released after high temperature lysis of high-LET irradiated cells decreased with repair-time and after 1–2 h repair, a large fraction of DSB was still unrejoined, but there was no significant contribution of heat-released DSB.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary data and Supplementary Figure 1 available online at http://informahealthcare.com/abs/doi/10.3109/09553002.2015.1068463.