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Processing of clustered DNA damage generates additional double-strand breaks in mammalian cells post-irradiation

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ABSTRACT

Clustered DNA damage sites, in which two or more lesions are formed within a few helical turns of the DNA after passage of a single radiation track, are signatures of DNA modifications induced by ionizing radiation in mammalian cells. Mutant hamster cells (*xrs*-5), deficient in non-homologous end joining (NHEJ), were irradiated at 37°C to determine whether any additional double-strand breaks (DSBs) are formed during processing of γ-radiation-induced DNA clustered damage sites. A class of non-DSB clustered DNA damage, corresponding to ~30% of the initial yield of DSBs, is converted into DSBs reflecting an artefact of preparation of genomic DNA for pulsed field gel electrophoresis. These clusters are removed within 4 min in both NHEJ-deficient and wild-type CHO cells. In *xrs*-5 cells, a proportion of non-DSB clustered DNA damage, representing ~10% of the total yield of non-DSB clustered DNA damage sites, are also converted into DSBs within ~30 min post-γ but not post-α irradiation through cellular processing at 37°C. That the majority of radiation-induced non-DSB clustered DNA damage sites are resistant to conversion into DSBs may be biologically significant at environmental levels of radiation exposure, as a non-DSB clustered damage site rather than a DSB, which only constitutes a minor proportion, is more likely to be induced in irradiated cells.

INTRODUCTION

DNA is an important target for the damaging effects of ionizing radiation and endogenously induced reactive oxygen species. Ionizing radiation produces through direct and indirect effects a variety of DNA lesions, such as single-strand breaks (SSBs), double-strand breaks (DSBs), AP sites (either apyrimidinic or apurinic), a variety of base modifications, sugar modifications (1–3), and DNA–DNA and DNA–protein cross-links. The DSB is generally thought to be the main lesion involved in cell killing and formation of chromosomal aberrations (4–6). Many of the lesions induced by ionizing radiation are chemically similar to those induced as by-products of oxidative metabolism; however, ionizing radiation also induces complex damage known as non-DSB clustered DNA damage sites (defined as two or more elemental lesions formed within one or two helical turns of DNA by a single radiation track) (7–9). It has been hypothesized that lesions within these complex DNA damage sites induced by ionizing radiation would be less readily repaired than when present as isolated lesions (10). Indeed the efficiency of repair of DSB induced in mammalian cells is critically dependent upon the ionization density of the radiation (11–13), which influences the complexity of DSB (10) and loss of genome stability. More recently, it has become evident that processing of lesions within non-DSB clustered DNA damage sites based on *in vitro* biochemical approaches is indeed retarded (14–24).

The prediction that radiation induces non-DSB clustered DNA damage sites initially arose from biophysical models (25,26). However, more recently experimental evidence has shown that non-DSB clustered damage sites are indeed induced in mammalian cells by γ-radiation in yields that are four to eight times that of prompt DSB (7,8,27). Therefore, at environmental levels of low LET radiation, even though the number of non-DSB clusters is low compared with the higher levels of endogenous single lesions present, they may be biologically significant against this background of readily repairable endogenous damage. For instance, non-DSB clustered DNA damage present in synthetic oligonucleotides is known to give higher levels of mutations than that of the individual lesions in *Escherichia coli*, a reflection of their reduced repairability leading to stalled replication (28–31). In fact, there is evidence in prokaryote systems that non-DSB clustered damage may, in part, be converted into DSB post-irradiation (32).

γ-Radiation also produces a type of non-DSB clustered damage, which contains heat-labile sites, in 'naked' DNA (7,33,34) and cellular DNA (35,36). It was estimated that these types of clustered damage sites, which are converted into DSBs artefactually in preparation of genomic DNA for analysis using conventional pulsed field gel electrophoresis (PFGE) protocols, account for ~30–40% of the initial number of DSBs induced by γ-radiation in cells at 4°C (36). That these apparent additional DSBs arise from conversion of labile sites into DSBs by heat is consistent with a doubling of the yield of radiation-induced DSBs in naked DNA following a heat

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treatment at 37°C, when proteins are absent (33,36). For more densely ionizing α-particles the yield of DSBs induced in 'naked' DNA is independent of heat treatment (37,38).

Very little information is known about the processing of radiation-induced non-DSB clustered DNA damage induced in mammalian cells by ionizing radiation. To optimize observation of any additional DSB formed during enzymatic processing of radiation-induced DNA damage at 37°C, a cell line (*xrs*-5) deficient in non-homologous end joining (NHEJ) (39) was used to minimize DSB rejoining. The findings were compared with those using the repair proficient parental hamster cell line. We present here the first evidence that a proportion of non-DSB clustered DNA damage sites are indeed converted post-irradiation into DSBs in mammalian cells through cellular processing.

MATERIALS AND METHODS

Cell culture

CHO-K1 cells were obtained from the European Tissue Culture Collection, V79-4 cells are fibroblasts derived from the Chinese hamster ovary (CHO) cell line, and *xrs*-5 cells, which are deficient in the NHEJ repair pathway, were derived from CHO-K1 cells. All cell types were grown as monolayers in Eagle's minimal essential medium (MEM; Sigma) supplemented with 10% fetal calf serum (Mycoplex/PAA), 0.1% penicillin/streptomycin (Gibco) and 0.1% L-glutamine (Gibco) and maintained at 37°C in 95% air/5% CO_2.

Irradiations

Cells were maintained at a given temperature between 4 and 37°C during irradiation with either ^{60}Co γ-rays, at a dose rate of either 25 or 41.5 Gy min^{-1}, or α-particles using the MRC α-particle irradiator, described previously (11). The α-particle spectrum has a peak energy of 3.31 MeV. The absorbed dose rate, determined by flux measurements using CR-39 plastic track detectors (40), was 20 Gy min^{-1}. The cells were α-irradiated in the presence of PBS in glass-walled irradiation dishes (internal diameter 30 mm) with Hostaphan bases (thickness 0.35 mg cm^{-2}) as described previously (11). Irradiations with ^{60}Co γ-rays were performed under similar conditions with the exception that cells were attached to Falcon flasks that were placed on a Perspex shelf (thickness 12 mm) prior to irradiation at the given temperature. Following irradiation, the cells were either placed on ice or maintained at 37°C for given periods to allow any DSB rejoining to occur, then placed on ice. The cells were then processed for the presence of DSBs using the following methods.

Determination of radiation-induced DNA DSBs in mammalian cells

FAR assay. The fraction of activity released (FAR) assay allows detection of radiation-induced DSBs and formamido-pyrimidine-DNA glycolyase (Fpg)-revealed DSBs present in the whole genome of the cells. These protocols were described in detail in Gulston *et al.* (7). Briefly tritiated cells labelled with [^{3}H]thymidine were irradiated as described above. Agarose plugs containing ~2.5×10^4 irradiated cells/100 µl agarose were prepared and the cells lysed using proteinase-K lysis conditions [outlined in Gulston *et al*. (7)], which involve maintaining the lysis conditions at 37°C for 24 h. For determination of Fpg-revealed DSBs, plugs containing the cells were lysed as above and then suspended in 400 µl of 1.25× endonuclease reaction buffer for 1 h at room temperature. Plugs were then re-suspended in fresh 1.25× endonuclease reaction buffer containing the optimum concentration of Fpg enzymes of 60 ng/µg DNA (7). Reactions were incubated at 37°C for 60 min.

These plugs were subjected to PFGE (Bio-Rad model CHEF DR11) to allow damaged DNA to migrate away from undamaged DNA in an agarose gel (0.8%, Ultrapure; Gibco). The conditions of the PFGE run were 45 V with 60 min pulse times for 96 h at pH 8.3 and 16°C. After electrophoresis the gel was stained for 1 h with 0.01 µg ml^{-1} ethidium bromide. Using a UV transilluminator, the agarose gel containing the lanes of migrated DNA were sectioned into 3–4 mm pieces. The segments were placed in glass scintillation vials, 200 µl of 1 mol dm^{-3} HCl was added and the mixture then heated to melt the gel. When the vials had cooled, 3 ml of liquid scintillant (Optiphase Highsafe 3) was added to each vial and mixed thoroughly by vortexing. The activity of ^{3}H migrating away from the well in each lane was determined using a scintillation counter (Beckman LS6500).

Determination of FAR. The yield of DSBs after irradiation was determined from the amount of DNA extracted from the well:

$$FAR = \frac{dpm(lane)}{dpm(lane) + dpm(well)} \qquad \mathbf{1}$$

The percentage of activity released is given by FAR × 100. The value of FAR was converted into the yield of DSBs/cell/Gy using the following equation:

$$F_{\mathrm{RETAINED}} = \exp^{[-\eta D(k/m)]}\{1+-\eta D(k/m)[1-(k/m)]\} \qquad \mathbf{2}$$

The experimentally determined $F_{\mathrm{RETAINED}} = 1 - \mathrm{FAR}$, η is the mean frequency of DSB per chromosome per unit dose, k is the exclusion size of DNA able to leave the well (≥5.7 Mb), m is the mean size of a chromosome. The value of m was taken to be 245 Mb for hamster cells and the number of chromosomes to be 22.

Hybridization assay. In this assay, detection of radiation-induced DSB was measured in a defined large fragment of the genome (41). The protocol is outlined in Gulston *et al.* (7). Briefly, cells grown as monolayers in T25 flasks were irradiated with ^{60}Co γ-rays as above and suspended in agarose plugs as described for the FAR assay, except that the cell density was ~0.5×10^6 irradiated cells/100 µl agarose.

Cells were lysed in the plug at 50°C for 48 h with a solution containing proteinase-K, *N*-lauroyl sarcosine and EDTA. Plugs were then washed twice in TE buffer and were suspended in 1.25× MluI restriction enzyme buffer [7.5 mM Tris–HCl (pH 7.9), 187.5 mM NaCl, 7.5 mM $MgCl_2$, 1.25 mM DTT and 0.125 mg/ml BSA] for 1 h at room temperature. Each 100 µl agarose plug was placed in 400 µl of fresh buffer

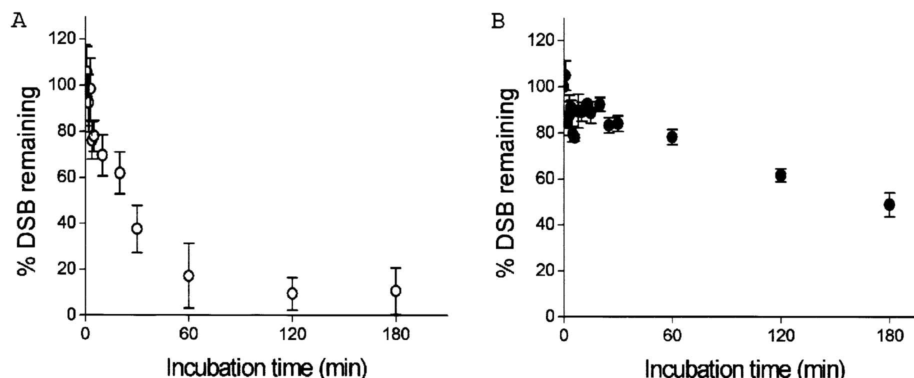

Figure 1. Overall rejoining of DSBs induced in (**A**) V79-4 wild-type hamster cells and (**B**) *xrs*-5 cells γ-irradiated at 37°C with a dose of 15 Gy delivered in 1 min and subsequently incubated at 37°C for up to 180 min. The yields of DSBs determined using the FAR assay are presented as percent breaks remaining after normalizing the initial yield to 100%.

containing 20 units of MluI restriction enzyme (Promega) and incubated at 37°C overnight. The DSB yield was determined using PFGE and Southern blotting as previously described (7). The PFGE running conditions were: 0.8% agarose, 1× TAE buffer gel, 2 V/cm in 1× TAE buffer, pH 8.3, with 50–5000 s switch times for 120 h at 12°C. The yeast molecular weight markers *Schizosaccharomyces pombe* (3.5–5.7 Mb) and *Saccharomyces cerevisiae* (0.2–2.2 Mb) were also run on each gel. After electrophoresis, DNA within the gel was stained with ethidium bromide, viewed over a UV transilluminator and photographed. The DNA in the gel was partially depurinated with HCl, rinsed in water and transferred onto a nylon membrane (Hybond-N+; Amersham) using a vacuum blotter in the presence of denaturing/transfer solution (0.5 M NaOH). The membrane was prepared for hybridization of the probe as described previously (7). The probe (ATCC 77273), specific for the 1.8 Mb MluI fragment of interest, was radioactively labelled with ^{32}P according to the manufacturer's recommendations (Amersham rediprime II random primer labelling system) to a specific activity of 1–2 × 10^9 c.p.m./µg probe. Hybridization of the probe was performed overnight at 65°C in pre-hybridization buffer. The membrane was washed, wrapped in saran wrap and placed in a phosphoimager screen for 1–5 days. The image was captured by a phosphoimager (Bio-Rad molecular imager FX) and analysed using Quantity One (Bio-Rad) software. The yield of DSBs (DSB/fragment) was determined using equation **3**, assuming a Poisson distribution of DSBs in the DNA. The equation depends on the intensity of activity associated with the specific 1.8 Mb band compared with the intensity of the whole lane, relative to that in the control, non-irradiated DNA lane:

$$\text{DSB yield} = -\ln[(I_{\text{Band}} / I_{\text{Lane}})_{\text{D}} / (I_{\text{Band}} / I_{\text{Lane}})_{\text{control}}] \qquad \mathbf{3}$$

where I_{Band} is band intensity, I_{Lane} is intensity in the entire lane, D is dose and control is non-irradiated DNA.

In the experiments reported, the error bars represent the SEM values calculated for each data point from at least three independent experiments.

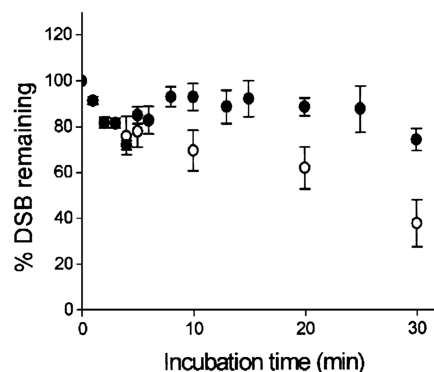

Figure 2. Overall rejoining of DSB in V79-4 (open circles) and *xrs*-5 (filled circles) cells γ-irradiated with a dose of 15 Gy delivered in <1 min and subsequently incubated at 37°C for up to 30 min. The yields of DSB measured using the FAR assay are presented as percent breaks remaining after normalizing the initial yield to 100%.

RESULTS

Global repair of γ-radiation-induced DSBs throughout the genome

The majority of studies to date on the time course of rejoining of radiation-induced DSBs are based on irradiation of the cells at 4°C followed by incubation at 37°C for given times. Additionally, the first time point for repair has generally been determined 10–15 min post-irradiation, whereas in the present study, particular care has been taken to determine repair at times <10 min, necessitating irradiation within 1 min and at 37°C. Figure 1A and B present the time dependence for the overall rejoining of DSBs induced in either V79-4 cells or *xrs*-5 cells γ-irradiated with 15 Gy within 1 min at 37°C followed by incubation at 37°C for up to 3 h post-irradiation. The initial yield of DSBs detected with both cell lines, corresponding to 27 DSBs/cell/Gy based on a FAR value of 13%, is similar to that determined for irradiation of V79-4 cells at 4°C (7), indicating that few detectable DSBs (<15%) have been rejoined within the irradiation period at 37°C.

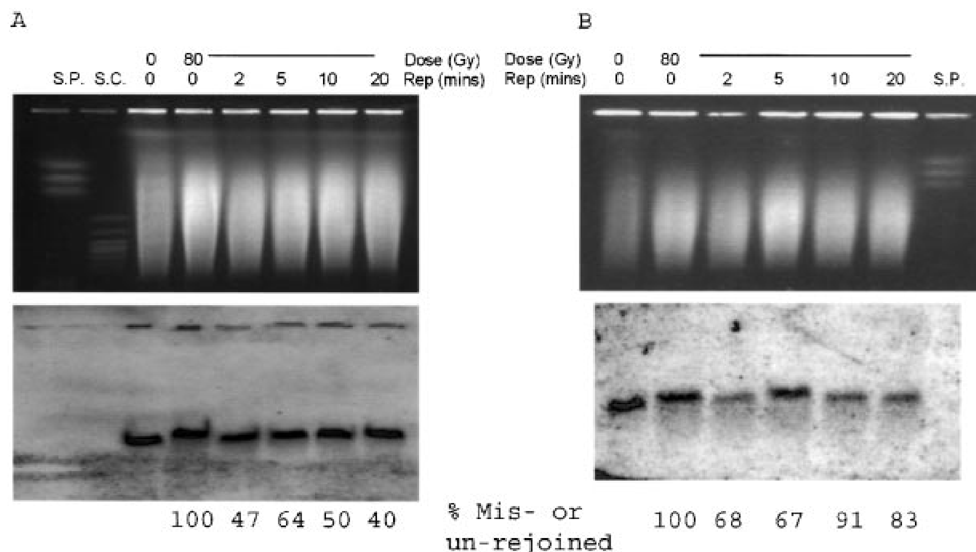

Figure 3. Correct rejoining measured using the hybridization assay for wild-type hamster (**A**) and *xrs*-5 (**B**) cells irradiated at 4°C with a dose of 80 Gy of ^{60}Co γ-radiation. In (A) and (B) the pulsed field gel of irradiated, lysed, MluI-restricted DNA was stained with ethidium bromide (top) and blotted onto nylon membrane for hybridization to probe ATCC 77273 (bottom). S.P. and S.C. are *S.pombe* and *S.cerevisiae* chromosomes as size markers, respectively.

Furthermore, the timescale for rejoining of DSBs induced in V79-4 cells (or CHO-K1 cells; data not shown) over 3 h is similar for irradiations at 37°C within 1 min or at 4°C (7). With *xrs*-5 cells the number of DSBs present at 3 h is >50%, reflecting the poor DSB rejoining efficiency associated with this mutant line. However, it is apparent that even with *xrs*-5 cells, a considerable percentage of initially detected DSBs are rejoined within 5 min.

For clarity, the time dependence of the number of DSBs present during the first 30 min following γ-radiation of wild type V79-4 and *xrs*-5 cells are presented in Figure 2. It is immediately apparent that the number of DSBs rejoined in the whole genome of V79-4 and *xrs*-5 cells differ significantly over the initial 30 min, although the profile of repair is remarkably similar within the initial 5 min with both cell lines. It is evident that during this initial 5 min, 25–30% of the initially detected DSBs have been removed by what would appear to be a very rapid repair process (see later). After this initial 5 min, the numbers of DSBs increase over the next 5–10 min at 37°C to a peak value of 93% of the initial yield but only with the DSB repair-deficient *xrs*-5 cells. With wild-type cells, the yield of DSBs continues to decrease with time.

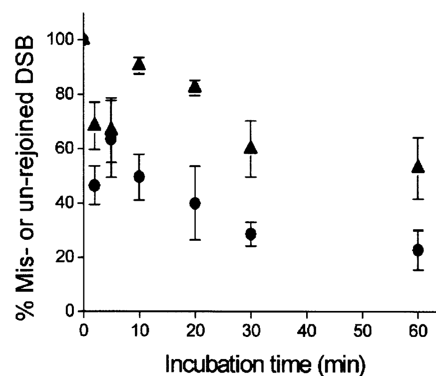

Figure 4. Percentage of mis- or un-rejoined DSBs measured using the hybridization assay in wild-type CHO-K1 (circles) and *xrs*-5 (triangles) cells irradiated at 4°C with a dose of 80 Gy of ^{60}Co γ-radiation and subsequently incubated at 37°C for various times up to 60 min. Yields are presented as percent mis- or un-rejoined DSBs remaining after normalizing the initially induced yields to 100%.

Fidelity of repair of γ-radiation-induced DSBs in a 1.8 Mb fragment of the genome

The primary purpose of employing the hybridization assay to determine DSB yields after repair incubation was to obtain a measure of the fidelity of the repair of radiation-induced DSBs, since correctly rejoined DSBs reconstitute the correct length restriction fragment. Therefore, any discrepancy between gross rejoining, measured using the FAR assay, and correct rejoining, measured by the hybridization assay, is a measure of repair fidelity.

Figure 3 shows representative examples of ethidium bromide stained gels following PFGE and the subsequent hybridization of radio-labelled probe to blots of these gels for CHO-K1 (Fig. 3A) and *xrs*-5 cells (Fig. 3B) which had been irradiated at 4°C with 80 Gy followed by a post-irradiation incubation at 37°C for the times shown. Using equation **3**, the yields of DSB detected immediately after irradiation at 4°C using the hybridization assay were calculated to be 28.1 ± 5.3 and 22.1 ± 3.2 DSBs/cell/Gy for CHO-K1 and *xrs*-5 cells, respectively. The time dependence of the yield of DSBs on post-irradiation incubation at 37°C in both cell lines is presented in Figure 4. It is clear that the DSBs removed within the initial 5 min (see also Fig. 2) are repaired with high fidelity in both cells lines and the yields of DSBs removed within 5 min correspond to 30–35% of the initial yield. After this initial 5 min, the numbers of DSBs increase over the next 5–10 min of incubation at 37°C to a peak value of 90% of the initial yield, but this is only seen with the DSB repair-deficient

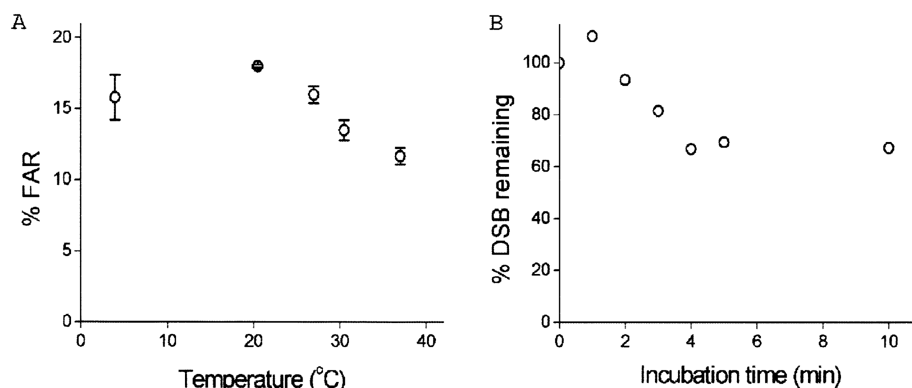

Figure 5. (**A**) Yield of DSBs expressed as percent FAR in V79-4 cells γ-irradiated with 15 Gy at different temperatures and (**B**) time for rejoining of DSBs induced by γ-irradiation of V79-4 cells with 18 Gy delivered within 0.5 min and at 37°C.

xrs-5 cells. This increase in DSBs corresponds to a yield of ~6 DSBs/cell/Gy. Subsequently, a proportion of the DSBs is then rejoined with high fidelity so that the yield of mis-rejoined or persistent DSBs reduces to 53 ± 11% after 1 h. With wild-type cells, the yield of mis-repaired or persistent DSBs continues to decrease with time, reaching a yield of 23 ± 7% at 1 h post-irradiation.

Temperature dependence of the yield of DSBs induced by γ-radiation

Since several studies have indicated that apparent additional DSBs may arise from conversion of labile sites into DSB by heat (see Introduction) and that a class of γ-radiation-induced DSBs are rejoined with high fidelity within 5 min (see above), the temperature dependence of the yield of DSB induced by γ-radiation of V79-4 cells was determined. Figure 5A shows the yield of induced DSB, expressed as percent FAR, as a function of the temperature of irradiation with a dose of 15 Gy delivered within 5 min. The yield of DSBs starts to decrease when the irradiation temperature is >20°C and at 37°C reaches a value which is 30% lower than that determined at 4°C. This reduction is consistent with that seen for the proportion of DSBs that are 'repaired' within 5 min, as shown in detail in Figure 5B when V79-4 cells are irradiated at 37°C within 0.5 min. That apparent additional DSBs may arise from conversion of labile sites into DSBs by heat is consistent with the rapid repair seen within 4 min. The artefactual conversion of a type of clustered DNA damage into DSBs in the preparative procedures (see Materials and Methods) for analysis of DSBs in DNA by conventional protocols for PFGE (35,36) was addressed using a salt lysis condition at 4°C followed by electrophoresis at 16°C (7) following irradiation of the cells. The yield of DSBs induced by γ-irradiation (20–100 Gy) using the salt lysis at 4°C is significantly lower by 25–30% than that using the conventional lysis conditions involving a prolonged period at 37°C (Supplementary Material, Fig. S1).

Rejoining of DSBs induced in V79-4 cells by α-particles at early times

Since 30–35% of DSBs induced by γ-radiation are removed within 5 min post-irradiation and the majority of DSBs induced by α-particles have been shown to be less repairable

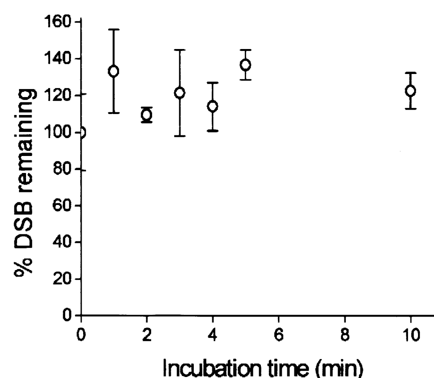

Figure 6. Overall rejoining of DSBs in V79-4 cells α-irradiated with a dose of 20 Gy delivered in <1 min at 37°C and subsequently incubated at 37°C for up to 10 min. The yields of DSBs measured using the FAR assay are presented as percent breaks remaining after normalizing the initial yield to 100%.

(11) and not inducible by heat (37,38) in 'naked' DNA, we investigated whether the rapid removal of DSBs, seen with γ-irradiation, depends on radiation quality. Figure 6 shows that the yield of DSBs induced in V79-4 cells by high LET α-radiation (20 Gy) remains essentially constant over the initial 10 min period at 37°C following irradiation. This persistence of DSBs induced by α-radiation is in contrast to the apparent loss of DSBs over 4 min seen with γ-radiation (Fig. 5B).

Since the yield of non-DSB clustered damage sites induced by γ-radiation is greater than that of prompt DSBs (7,27), the induction of clustered damage by α-radiation was investigated using Fpg to reveal these types of damage sites as described previously (7). With α-radiation (20 Gy), a post-irradiation treatment of extracted genomic DNA with Fpg does not result in an increase in the yield of additional enzymatically induced DSBs, indicative of few if any non-DSB clustered DNA damage sites being converted into DSBs. This apparent absence of enzymatically induced DSBs is in contrast to the formation of an extra 1.1 DSB for each prompt DSB (including heat-labile sites) in γ-irradiated V79-4 cells following a treatment with Fpg (7).

DISCUSSION

By irradiation of mammalian cells at 37°C and the use of *xrs*-5 cells to minimize DSB rejoining, the visualization of certain types of clustered DNA damage in cells has been optimized. Evidence is presented showing that: (i) a type of non-DSB clustered DNA damage site(s) is converted, post-γ irradiation, into DSBs during their intracellular, enzymatic processing in mammalian cells and (ii) a type of γ-radiation-induced clustered DNA damage containing a heat-labile lesion(s) is repaired with high fidelity within 4 min.

It is proposed that these damage sites, visualized as DSBs (see Figs 2–4), represent two types of non-DSB clustered DNA damage. In contrast to these observations with γ-radiation, additional cellular DSBs resulting from heat-labile sites or conversion of Fpg-sensitive clustered sites into DSBs are not evident following α-irradiation. As discussed previously, these differences reflect differences in the ionization density of the radiation, which determine differences in the complexity of radiation-induced damage (37), and that the majority of damage induced by α-radiation arises through direct energy deposition events in the DNA (41) with no additional DSBs being produced through a heat treatment at 37°C.

Fast component of DSB repair-clustered sites containing heat-labile sites

The timescale for rapid removal of clustered sites containing heat-labile sites and seen as DSBs (within 5 min) at 37°C is the same in both NHEJ-proficient and -deficient CHO cell lines. Furthermore, a rapid post-irradiation loss of DSBs corresponding to 20–35% of the overall yield of DSBs was seen within 15 min, measured using PFGE with DT40 cells proficient in DSB repair, DT40 mutant cells deficient in homologous recombination (HR), NHEJ or both (42) and also BRCA2-deficient cells (43). Therefore, this rapid removal of DSBs either represents a class of radiation-induced DSBs which are repaired rapidly by processes that are independent of NHEJ, HR or both, or is an artefact of the PFGE assay used to measure DSBs. Recently, the rapid rejoining of DSBs induced in cells deficient in XRCC5 was not seen if a protocol involving cold lysis was used (36). Therefore, it is suggested that these apparent DSBs, which are rapidly repaired with high fidelity, are rarely, if at all, converted into DSBs through natural processing in cells. Therefore, we favour the formation of clustered DNA damage sites that contain either bistranded heat-labile sites and/or an SSB [formed directly or through base excision repair (BER) processing of a base lesion] opposed to a heat-labile site. If the heat-labile lesion(s)/SSB is still present prior to its repair in a clustered damage site, it may be trapped and converted into DSBs during the cell lysis step at elevated temperatures in the preparation of genomic DNA for analysis by PFGE. Radiation-induced damage sites containing heat-labile lesions which are converted into DSBs have previously been detected during induction at 4°C using low temperature lysis (35,36) and it was verified in the present study that low temperature lysis conditions do result in fewer DSBs. It was suggested that the additional DSBs reflect an artefact of the preparation of genomic DNA for PFGE. Consistent with this suggestion is the observation that even with naked DNA irradiated at 4°C when enzymes are absent, additional DSBs (7,33,34), which represent a doubling of the yield of DSBs, are formed with a half-life of ~1 h (33,35) if the irradiated DNA is kept at 37°C post-irradiation for several hours. The time required to convert heat-labile sites or clusters containing SSB/heat-labile sites into DSBs during lysis of irradiated cells at 50°C is ~10 h (35,36), whereas at ⩽20°C, heat-labile sites remain intact (36). These additional DSBs are therefore induced in cells on a time scale which is long relative to that of the 5 min for removal of the precursor site, which if trapped leads to DSBs. If the heat-labile cluster site contains a SSB and a heat-labile site, the timescale for removal of this cluster would be consistent with the timescale of SSB rejoining in cells (44). 2′-Deoxyribonolactone, formed by C1′ hydrogen abstraction, is one of the potential precursors to an SSB through heat treatment or mild alkali treatment (45).

The presence of these heat-labile clustered DNA damage sites, revealed as DSBs by PFGE assays, may have significant consequences when evaluating the kinetics of repair of DSBs. For instance, the generally observed fast repair of a proportion of DSBs when cells are irradiated at 4°C would appear slower than the 5 min repair period determined in this study, since time would be required for the cells to reach equilibrium at 37°C post-irradiation. As seen in Figure 5A, even at 30°C the majority of the heat-labile clustered sites still persist.

An additional consideration relates to quantification of the fidelity of repair of DSBs using the hybridization assay (7,46–48). Generally, cells have been irradiated at 4°C prior to a post-irradiation incubation at 37°C. In the majority of these studies, including those with repair-deficient mutants, a proportion of DSBs are repaired rapidly with high fidelity. For instance, it was shown that half of the DSBs, corresponding to ~30% of the total yield of DSBs, are repaired with high fidelity in 9 min (47). This yield is similar to the yield determined in the present study for heat-labile clustered sites repaired with high fidelity in both mutant and wild-type CHO-K1 cells at early times.

Additional component of DSBs in *xrs*-5 cells clustered damage sites containing enzyme sensitive lesions

With *xrs*-5 cells it has been possible to detect a second type of γ-radiation-induced clustered damage through visualization of their conversion into additional DSBs intracellularly (see Fig. 2). The yield of these clustered damage sites represents only a small fraction of the overall yield of clustered damage sites induced in cells by γ-radiation (7,27). In fact, in prokaryote systems some non-DSB clustered damage sites have been shown to be converted through intracellular processing into DSB post-irradiation (32). Although we are unable to identify the lesions within the cluster, potential candidates for conversion of clustered damage sites into DSBs are two closely opposed AP sites. For instance, two closely spaced, bistranded abasic (AP) sites or an AP site close to an opposed SSB do yield a DSB as shown using synthetic clustered damage within oligonucleotides (2,3,30,49). The accumulating evidence indicates that clustered damage sites, which do not contain an AP site, are not converted into DSBs by BER (14–21,24). An alternative explanation for formation of additional DSBs involving peroxide generated during irradiation is unlikely at the doses used, as the concentration of peroxide is estimated to be an order of magnitude lower than that reported to induce SSB in cells through treatment

with hydrogen peroxide (50). DSBs are induced only at very high concentrations of hydrogen peroxide (>0.02 M) (50).

That 'additional' DSBs are not seen following α-irradiation (data not shown) reflects the greater complexity of the clustered sites as emphasized by the lack of visualization of clusters following a post-irradiation treatment with Fpg. Similar differences were seen with γ- and α-irradiated hydrated DNA (34,37), when it was inferred that the clustered DNA damage sites induced by α-radiation are sufficiently complex to inhibit their enzymatic conversion into DSBs in contrast to the enzymatically revealed DSBs seen with γ-irradiation.

In conclusion, non-DSB clustered DNA damage sites are found in cells and a proportion are converted post-γ but not post-α irradiation into DSBs through their cellular processing. While damage such as DNA DSBs is commonly considered to be one of the most harmful effects of irradiation, at environmental levels of radiation exposure the probability that a clustered damage site and not a DSB may be induced in any one cell becomes important, especially in the light of their increased mutagenic effects relative to that of single lesions.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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