Repair of radiation-induced heat-labile sites is independent of DNA-PKcs, XRCC1 or PARP

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Ionizing radiation induces a variety of different DNA lesions: in addition to the most critical DNA damage, the DSB, numerous base alterations, SSBs and other modifications of the DNA double-helix are formed. When several non-DSB lesions are clustered within a short distance along DNA, or close to a DSB, they may interfere with the repair of DSBs and affect the measurement of DSB induction and repair. We have previously shown that a substantial fraction of DSBs measured by pulsed-field gel electrophoresis (PFGE) are in fact due to heat-labile sites (HLS) within clustered lesions, thus reflecting an artifact of preparation of genomic DNA at elevated temperature. To further characterize the influence of HLS on DSB induction and repair, four human cell lines (GM5758, GM7166, M059K, U-1810) with apparently normal DSB rejoining were tested for bi-phasic rejoining after gamma irradiation. When heat-released DSBs were excluded from the measurements the fraction of fast rejoining decreased to less than 50% of the total. However, neither the half-times of the fast ($t_{1/2}$ = 7-8 min) or slow ($t_{1/2}$ = 2.5 h) DSB rejoining were changed significantly. At t=0 the heat-released DSBs accounted for almost 40% of the DSBs, corresponding to 10 extra DSB/cell/Gy in the initial DSB yield. These heat-released DSBs were repaired within 60-90 min in all tested cells, including M059K cells treated with wortmannin or DNA-PKcs defect M059J cells. Furthermore, cells lacking XRCC1 or Poly(ADP-ribose) polymerase-1 (PARP-1) rejoined both total DSBs and heat-released DSBs similar to normal cells. In summary, the presence of heat-labile sites have a substantial impact on DSB induction yields and DSB rejoining rates measured by pulsed-field gel electrophoresis, and HLS repair is independent of DNA-PKcs, XRCC1 and PARP.

INTRODUCTION

The DNA double-strand break (DSB) is considered the most critical DNA damage induced by ionizing radiation. However, it is believed that in addition to DSBs, numerous base alterations, single-strand breaks and other modifications of the DNA doublehelix are formed within multiply damage sites. Much of our knowledge about radiation-induced DSB and repair in mammalian cells comes from studies utilizing the techniques of constant field or pulsed-field gel electrophoresis (PFGE). The preparation of protein-free genomic DNA for electrophoresis typically includes a cell lysis step in which agarose-embedded cells are incubated at 50 ºC in a lysis buffer (*1*) and it has been shown that radiation-induced heat-labile sites produced in naked DNA (*2, 3*) or in intact cells (*4, 5*) can be converted into DSB in this procedure. The exact nature of heat-labile sites generated by irradiation is not known but radiation damage to sugar-phosphate residues, e.g. from attack by OH radicals, are potential labile sites that could be converted into strand breaks upon heating (*4*). Thus double-stranded heat-labile sites and/or an SSB opposed to a heat-labile site could be converted into a DSB during the cell lysis step at elevated temperatures in the preparation of naked genomic DNA for analysis by PFGE. These artifactual DSBs may affect both induction yields and estimates of DSB repair (*4-6*).

By preparing genomic DNA without the release of heat-labile sites (HLS) it was shown that initial DSB yields, obtained by conventional warm lysis (e.g. at 50ºC), are overestimated by 30-40% (*5*) in mammalian cells exposed to low-LET radiation. Furthermore, the repair kinetics in terms of fast and slow rejoining of DSBs has been linked to different repair processes (*7*) and the presence of artifactual DSBs will most likely affect these parameters. This is further accentuated in cells with defects in the repair capacity: the presence of heat-labile sites which are repaired independently of DSB repair pathways could give a false impression of DSB repair. Indeed it was shown that in cell mutants with defects

in non-homologous end joining (NHEJ), the apparent fast rejoining of DSB was almost exclusively due to the repair of HLS (*5, 6, 8*).

To further study the effects of radiation-induced HLS on the analysis of DSB induction and repair in irradiated mammalian cells we used a protocol to prepare naked DNA without releasing heat-labile sites (*5*). We here show that the presence of HLS increases the initial DSB yield by almost 40% in warm-lysis conditions and that the removal of these artifactual DSBs decreases the fraction of fast rejoining to less than 50%. Furthermore, cells with inhibited kinase activity of DNA-PKcs are shown to be totally unable to rejoin radiation-induced DSBs within the first hour after irradiation. The apparent fast rejoining normally seen in cells without DNA-PK activity is therefore completely due to repair of HLS. We also show that the lesions involved in the release of heat-labile sites into DSBs are repaired independently of XRCC1 and PARP-1.

MATERIALS AND METHODS

Cells and culture conditions

Normal human skin fibroblasts GM5758 and NBS1 defective human fibroblasts GM7166 (Human Genetic Mutant Cell Repository, Camden, NJ, USA) were grown in Eagle minimal essential medium (MEM). Medium for GM5758 cells was supplemented with 2 × concentration of MEM vitamins and both non-essential and essential amino acids. Human tumor cells M059J and M059K (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco´s modified Eagle´s medium (DMEM) and Ham´s F12 nutrient mixture in 1:1 ratio supplemented with 1×concentration non-essential amino acids. Non-small cell lung carcinoma cells U-1810 (*9*) were grown in RPMI 1640 medium. The hamster cell lines AA8 (wild-type) and EM9 (XRCC1 mutant), and mouse fibroblasts A11 (PARP1

mutant) were grown in DMEM. The cell culture medium for all cell lines was supplemented with 10-15% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin. The cells were maintained in 25 cm^2 cell culture flasks at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells for PFGE were plated in 3-cm dishes and labelled with 1000 Bq/ml [methyl-^{14}C] thymidine (Amersham Bioscience, Uppsala, Sweden) for approximately two doubling times. Before irradiation the ^{14}C activity was chased with medium without radioactivity.

Chemicals and irradiation

Cells were treated with complete medium containing 50 µM of the DNA-PK inhibitor wortmannin (Sigma-Aldrich) or 5 mM of the PARP inhibitor 3-AB (3-aminobenzamide, Sigma-Aldrich). Cells were preincubated with drugs 1.5 h before irradiation. The drugs were present during irradiation and for the stated repair times. Fresh medium containing wortmannin or 3-AB was added directly after irradiation.

Cells were irradiated with ^{137}Cs γ-ray photons (Gammacell 40 Exactor, MDS Nordion, Kanata, Canada) at a dose-rate of 1.25 Gy per minute. The cells were put on ice 20-30 minutes before irradiation and were kept on ice during the entire irradiation.

Pulsed-field gel electrophoresis

After irradiation and repair incubation at 37°C, cells were trypsinized and mixed with low gelling-point agarose (InCert, BMA, Rockville, MD) to a final concentration of 1.5-2.5 x 10^6 cells/ml in 0.6% agarose. The stated repair times spans from the addition of fresh 37°C medium to the incubation of agarose plugs at 4°C. Thus there could be a delay in both the initiation and inhibition of the repair, which may introduce some uncertainty in the estimation of repair times. The mixture was transferred into plug-moulds (20-100 µl) and

allowed to set at 4ºC in 15-20 minutes. The plugs with cells were then transferred to 10 plug volumes of ESP lysis buffer at 4ºC (2% N-lauroylsarcosine (Sigma), 1 mg/ml Proteinase K (Roche), all diluted in 0.5 M EDTA (Na_3) at pH 8.0). After 1 hour at 4ºC the samples were treated either by a conventional warm lysis procedure (incubation at 50ºC for 18-22 hours), or by a novel cold two-step protocol to prepare naked chromosomal DNA without releasing heat-labile sites (*5*). In the cold two-step protocol the plugs are kept in cold ESP (4ºC). After >20 hours the ESP buffer was removed and replaced with 20 plug volumes HS-buffer and incubated overnight at 4ºC (HS: High Salt; 1.85 M NaCl, 0.15 M KCl, 5 mM $MgCl_2$, 2mM EDTA, 4 mM Tris, 0.5 % Triton X-100, pH 7.5, Triton X-100 is added just before use).

Plugs were washed 2 x 1 hour in 0.1 M EDTA and 1 x 1 hour in 0.5 x TBE at 4ºC prior to electrophoresis. The plugs were then loaded into wells in a chilled (4ºC) agarose gel (0.8% SeaKem Gold, BMA). The gel was placed into a PFGE unit (Gene Navigator, Amersham Pharmacia Biotech, Uppsala, Sweden) with 120° between the fields. DNA was separated by PFGE at 2 V/cm in TBE x 0.5 using five different phases (10, 20, 30, 40 and 60 minute pulses for 3, 5:20, 8, 9:20 and 20 hours respectively; total time 45 hours and 40 minutes). The temperature during electrophoresis was 11ºC. Following electrophoresis, the gels were sliced at the position of the 5.7 Mbp chromosome from *S. pombe* (BMA), and ^{14}C in the gel segments was measured by liquid scintillation. The fraction of radioactivity corresponding to DNA of size less than 5.7 Mbp was corrected for the maximum fraction of mobile DNA (100% for GM5758 and GM7166, 90% for M059J, M059K and U-1810, 75% for the hamster and mouse cell lines, (*10*)) and the number of DSBs per bp was then calculated using a random breakage model (*11*):

$$F = 1 - (1 + n\, d\, k\, (1 - k/X))\, \exp(-n\, d\, k) \qquad (1)$$

where F is the fraction of DNA with sizes less than the threshold k in bp (5.7×10^6 bp), n is the number of DSBs per Gy per bp, d is the radiation dose in Gy and X is the mean size of a chromosome in bp. The calculations are only slightly dependent on the exact chromosome size and $X = 1.3 \times 10^8$ bp (mean size of a human chromosome) was used for all cell lines. The equation is solved for n using Newton-Raphson iterative method.

RESULTS

Heat-labile sites affect the fast component of DSB repair

DSB measurements involving cell lysis at elevated temperatures (e.g. 37-50ºC) have the risk to release HLS into DSBs and therefore almost all data for DSB repair after exposure to low- or high LET radiation, obtained by PFGE measurements include measurement of heat-released DSBs (*4-6*). An important purpose of the present investigation was to further reveal how HLS affect the kinetics in DSB repair. To determine the influence of HLS on the rejoining kinetics, four cell lines with apparently normal DSB repair-kinetics were irradiated with γ-rays and assayed by PFGE using conventional warm lysis (50°C) or a new cold lysis protocol (*5*). The pooled results for normal human diploid fibroblast cell line GM5758, NBS1 defective human fibroblasts GM7166, human glioblastoma cell line M059K and human lung carcinoma cells U-1810 are shown in Fig. 1A. The warm lysis protocol gave a higher initial yield (at t = 0 h). However, after 60-90 minutes of repair the rejoining curves for the two lysis protocols coincides, indicating that all HLS are repaired. In Fig. 1B the data for the difference between warm and cold lysis are plotted, thus representing the repair of heat-released DSBs. Fitting of double-exponential curves to the data in Fig. 1A, representing bi-phasic rejoining, showed that the fraction of fast rejoining decreased from 0.56 for warm lysis conditions to 0.48 for cold lysis conditions. However, neither the half-times of the fast ($t_{1/2}$ = 7.4 min), nor the slow ($t_{1/2}$ = 2.5 h) DSB rejoining were changed (Table 1). The initial

number of DSBs induced by photons (t=0h) was 37.9 and 27.3 DSB/cell/Gy for the warm and cold lysis protocols, respectively (Table 1). Thus, the release of heat-labile sites after photon irradiation increases the initial DSB yield by almost 40%, corresponding to more than 10 extra DSB/cell/Gy (Table 1 and Fig. 1B).

No fast DSB rejoining in cells lacking DNA-PKcs activity

To further analyse the removal of HLS and how this affects the estimates of DSB rejoining, human M059K cells were incubated with the DNA-PK inhibitor wortmannin before and after irradiation with 40 Gy γ-rays. When the cold lysis protocol was used, apparently no fast DSB repair was present (Fig. 2A). In contrast, DSB analysis using the conventional warm lysis gave a higher initial DSB yield and a significant fast component of the repair. Almost identical results were obtained when DNA-PKcs defect M059J cells were used (Fig. 2B). Thus, the repair of HLS is independent of DNA-PKcs. Furthermore, cells deficient in DNA-PKcs, or wild-type cells treated with the DNA-PK inhibitor wortmannin, do not have a residual fast repair component when lysed with the cold lysis protocol.

Lack of XRCC1 or PARP-1 do not affect repair of heat-labile sites

The exact nature of HLS is not fully understood but it has been suggested that oxidative damage to sugar residues in DNA are potential break-sites upon heat-treatment (*4, 12, 13*). The presence of heat-released DSBs should then depend on both SSB repair (in cases where an SSB is opposed to a HLS on double-stranded DNA) and repair of HLS themselves. In Fig. 3, normal hamster cells and hamster or mouse cells defective in SSB repair were irradiated with 40 Gy and analyzed for their removal of HLS. Compared to wild-type AA8 cells (Fig. 3A), EM9 cells lacking functional XRCC1 (Fig. 3B) and A11 cells deficient in PARP-1 (Fig. 3C) did not display any delay in removal of heat-released DSBs.

Furthermore, normal human fibroblasts treated with the PARP-inhibitor 3-AB (3-aminobenzamide) responded in a similar way (data not shown). Thus, the repair of lesions involved in the release of heat-labile sites into DSBs is independent of XRCC1 and PARP-1.

DISCUSSION

After exposure to ionizing radiation several types of lesions are clustered on DNA. During treatment at elevated temperatures (37-50ºC), as in the preparation of naked genomic DNA in PFGE assays (*4, 5*), heat-labile sites on both strands, or on one strand and a SSB on the other strand, could be converted into a DSB. Once formed, these heat-released DSBs cannot be distinguished from the prompt DSBs formed directly by ionizing radiation. The conversion of HLS into DSBs is specific for cells exposed to ionising radiation, while other DSB-inducing agents such as *Hae*III restriction enzymes (*5*) or bleomycin (Stenerlöw *et al*. unpublished) do not lead to artifactual release of DSBs in PFGE preparations.

In the present investigation heat-labile sites released by warm lysis conditions were found to increase the initial DSB yield by 10-11 DSB/cell/Gy when analyzed in four human cell lines after gamma (^{137}Cs) irradiation (Table 1, Fig. 1). This result is in agreement with previous measurements for human fibroblast cells and human M059K cells (*5*). The contribution of HLS to the total DSB yield was similar in human M059K cells treated with wortmannin, DNA-PKcs defective M059J cells (Fig. 2), and three rodent cell lines (Fig. 3). Thus the release of HLS into DSBs leads to an overestimation of the initial yield of prompt DSB by almost 40% as measured by PFGE in mammalian cells after low-LET irradiation. From our data using cold lysis, i.e. a protocol to prepare naked genomic DNA without release of HLS, we conclude that about 27 prompt DSB/cell/Gy are induced in human diploid cells (Table 1). These data are supported by studies on γ-H2AX foci and analysis of

other repair related proteins, reporting 23-27 foci/cell/Gy (*14-18*) although other results have been obtained (*19*).

For densely ionizing radiation, data suggest that heat-released DSB may account for 15-20% of the total DSB after exposure to high-LET radiation (*20, 21*) or ^{125}I-decays within DNA (*22*).

Apart from affecting the initial DSB yield, artifactual release of HLS into DSBs also influences the estimates of DSB rejoining. Fitting of double-exponential curves, representing bi-phasic rejoining, gave half-times of 7.4 min and 2.5 h, for the fast and slow rejoining, respectively, and did not differ between the two lysis protocols. However, the fraction of DSBs rejoined by the fast phase decreased and was below 50% for the cold lysis protocol (Table 1). These data are in agreement with recent mathematical approaches to estimate repair of prompt DSBs (*23*). On the other hand, M059K cells treated with 50 μM wortmannin or M059J cells lacking functional DNA-PKcs did not show any fast DSB rejoining at all; the only fast rejoining was due to rejoining of HLS (Fig. 2), possibly explaining previous results showing fast rejoining in NHEJ mutants (*7, 24-29*). Our data are in agreement with recent experiments using DNA-PK deficient cells and show that the heat-labile sites are repaired by a non-DSB repair pathway without involving DNA-PK. Exclusion of HLS from the DSB measurement is therefore critical to obtain proper description of DNA damage and repair after irradiation. In fact, DNA-PK defective cells show very little DSB rejoining up to 4-20 hours after low- or high-LET irradiation (*5, 8*) and the number of γ-H2AX foci in M059J cells remain constant over 0-20 hours after radiation exposure (*8*), indicating that functional DNA-PK complex is critical for rejoining of broken DNA ends.

The exact nature of heat-labile sites generated by irradiation is not known but damage to sugar-phosphate residues, e.g. from attack by OH radicals, are potential labile sites that could be converted into strand breaks upon heating or alkali treatment (*4*). Thus double-

stranded heat-labile sites or an SSB opposed to a heat-labile site within a multiple damaged site (MDS) could be converted into a DSB during the cell lysis step at elevated temperatures in the preparation of naked genomic DNA. Repair mechanisms for sugar damage in general are not known. However, removal of ribose sugar from DNA in cell-free extracts is carried out by the enzymes RNaseH (class 2) and Fen1 (*30*). The resulting one-nucleotide gap is a potential substrate for DNA polymerase β and DNA ligase III in association with XRCC1, although it has not been proven that these enzymes are involved. At present it is not known how general this mechanism is for repair of other sugar modifications, but it is a possible mechanism involved in repair of HLS. It's dependence on PARP-1 and other secondary proteins is also not known. It should be pointed out in the present context of heat labile sites that already the initial nick, without further processing, will remove the site as an HLS in our assay and make it a DNA strand break instead.

The removal of HLS in our experiments was fast, and after 1-1.5 h repair there were no heat-released DSBs present in any of the eight tested cell lines (Fig. 1-3). The removal of heat-released DSBs in these cells suggest either a single phase with a half-time of 12 min, or two phases with half-times of 2 min and 20 min, respectively (data not shown). A fast kinetics with half-times of 2-3 min is within the lower range of 2-14 min reported for SSB repair (*31, 32*) and it is possible that longer half-times reflect the repair of HLS. The repair of single SSBs involves several proteins and both XRCC1 and PARP-1 have important roles in this process (*33*). PARP-1 rapidly binds the SSB and is believed to recruit the molecular scaffold protein XRCC1 to the break site where it interacts with ligase III (*33*). EM9 cells have a XRCC1 defect and a resulting 6-fold decrease in ligase III activity (*34*) but are still capable to repair SSBs, although at a lower rate than normal cells (*35-37*), perhaps by the use of a ligase I-dependent pathway (*33*). However, both EM9 cells and A11 cells (defective in PARP-1), removed potential heat-released DSBs at rates similar to wild-type AA8 cells (Fig.

3). Furthermore, normal human fibroblast cells GM5758 treated with the PARP inhibitor 3-AB, removed potential heat-released DSBs with similar kinetics as untreated cells (data not shown). Since the XRCC1 and PARP-1 proteins are important for SSB repair in general, these data suggest that SSB repair is not important in removing potential heat-released DSBs at sites containing a HLS opposite a SSB. There are data that suggests that SSBs within damage clusters are repaired with a slower rate than single SSBs (*38, 39*). Because of this, the rate-limiting step could be repair of the HLS, which may not require PARP-1 or XRCC1, but further studies are needed to confirm this hypothesis. There are also indications that the cell has capabilities to repair clustered lesions without causing DSBs (*40, 41*), although this sometimes fails (*42*). However, regardless of whether a DSB is induced as a consequence of the repair event or not, the heat-released DSB would be removed from detection in our assay, which compares the number of DSBs with or without heat treatment. In other words, we cannot distinguish whether the HLS was repaired or transformed into a new DSB (which would be disguised by simultaneous DSB rejoining at other sites).

In summary, the presence of heat-labile sites has a substantial impact on DSB induction yields and DSB rejoining rates measured by pulsed-field gel electrophoresis. Exclusion of HLS from these measurements reduces both the induction yield and the fraction of DSB rejoined by a fast kinetics. The lesions involved in the release of heat-labile sites into DSBs are removed independent of DNA-PKcs, XRCC1 or PARP-1. Importantly, cells with defect or inhibited function of DNA-PKcs do not show any fast rejoining of DSBs when HLS are eliminated.

ACKOWLEDGEMENTS

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Figure legends

FIG. 1. Rejoining of DSBs in four human cell lines GM5758, GM7166, M059K and U-1810 after 40 Gy γ-rays. (A) Cells were lysed using conventional warm protocol at 50ºC measuring total DSBs (open circles, broken line) or a cold protocol at 4ºC measuring prompt DSBs (filled triangles, solid line). Bi-exponential curves were fitted to the data. (B) Rejoining of heat-labile sites that are released as DSBs in conventional PFGE assay. Each data point represents the difference between the number of DSBs obtained by the conventional protocol and the cold protocol in A. Data are from 3-5 experiments for each cell line.

FIG. 2. Rejoining of DSBs in human cells without functional DNA-PKcs after 40 Gy γ-rays. (A) M059K (DNA-PKcs proficient) cells treated with 50 μM wortmannin. (B) M059J (DNA-PKcs deficient) cells. Cells were lysed using a conventional warm protocol at 50ºC (open circles, broken line) or a cold protocol at 4ºC (filled triangles, solid line) prior to PFGE analysis. Mean and SEM from 3-5 experiments.

FIG. 3. Rejoining of heat-labile sites that are released as DSBs in conventional PFGE assay. Cells were irradiated with 40 Gy γ-rays and incubated for repair. Cells were lysed using conventional warm protocol at 50ºC or a cold protocol at 4ºC. Each data point represents the difference between DSBs obtained by the warm protocol and the cold protocol. (A) Wild-type AA8 cells. (B) EM9 cells with a defect in XRCC1. (C) A11 cells with a defect in PARP-1. Bi-exponential curves were fitted to the data from 1-4 experiments.

TABLE 1

Double-strand break rejoning in human cell lines after exposure to γ-rays

Cell line	Conventional DNA extraction[a]				Cold DNA extraction[b]			
	$T_{1/2}$ fast (min)	$T_{1/2}$ slow (h)	F_{fast}	DSB/cell/Gy	$T_{1/2}$ fast (min)	$T_{1/2}$ slow (h)	F_{fast}	DSB/cell/Gy
GM5758	7.7	2.1	0.53	38.2	7.1	2.2	0.42	27.6
GM7161	7.8	2.0	0.57	36.7	8.9	2.4	0.53	27.8
M059K	5.0	2.7	0.59	37.5	5.2	4.4	0.51	26.4
U-1810	9.3	2.6	0.56	38.0	7.6	2.8	0.46	27.7
All cells[c]	**7.3 +/-0.8**	**2.3 +/-0.3**	**0.56 +/-0.03**	**37.9 +/-1.4**	**7.5 +/-1.2**	**2.7 +/-0.4**	**0.48 +/-0.04**	**27.3 +/-1.2**

[a] Extraction of DNA using conventional lysis at 50ºC (see Materials and Methods)

[b] Extraction of DNA using cold lysis at 4ºC (see Materials and Methods)

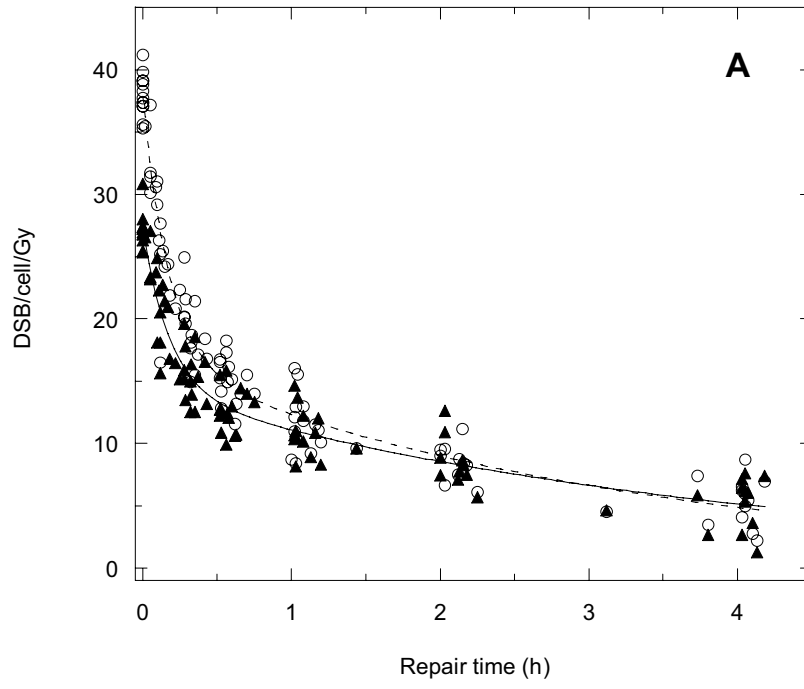[c] Based on curve-fitting to data points for all cell lines in Fig. 1A
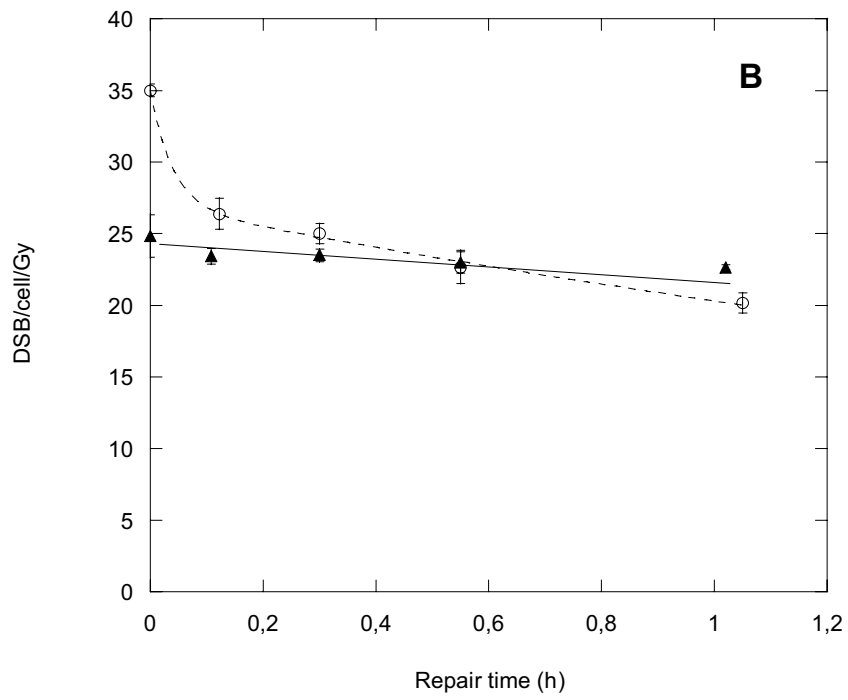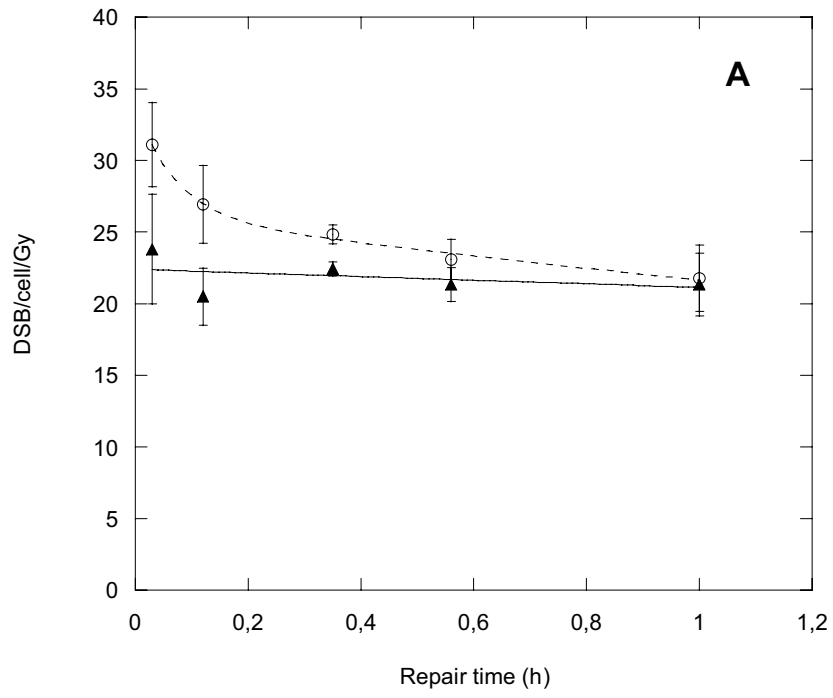
Figure 1
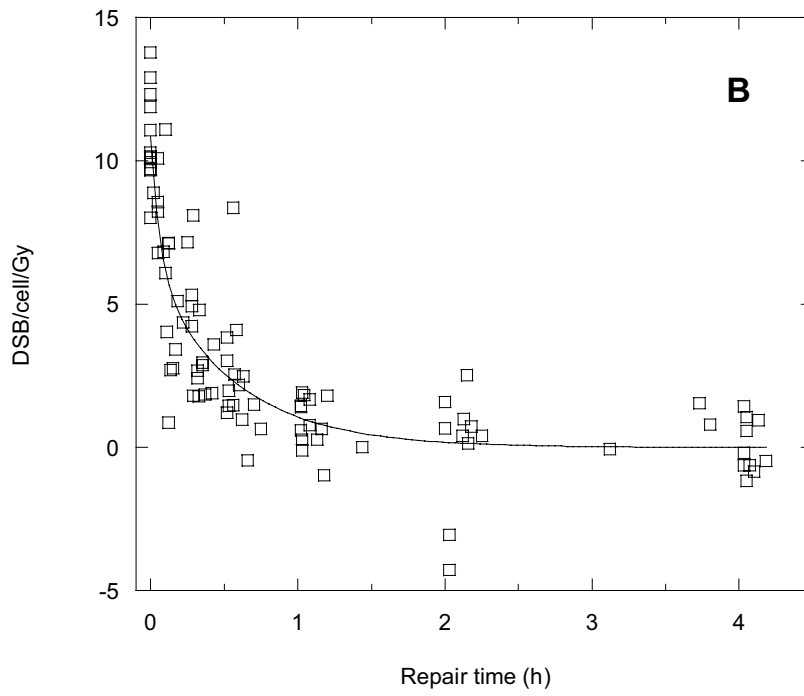
Figure 2

Figure 3

Fig. 3 (cont.)