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DNA repair after X-irradiation: lessons from plants

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Abstract

The effects of low-dose radiation causing DNA damage continue to be subjects of interest. Problems with existing approaches to low-dose DNA damage are that single-strand breaks (the predominant radiation-induced lesion) are very rapidly repaired and that results using current methods for measuring DNA damage can be difficult to interpret. As a novel approach, we conducted studies using plants (rye grass and the model plant *Arabidopsis*) exposed to X-rays and used the alkaline comet assay to measure DNA damage and repair after exposures. Consistent with previous studies, we detected so-called 'rapid' and 'slow' phases of DNA repair after acute exposures of 5 and 15 Gy. After exposures corresponding to 2 Gy and lower, 'rapid' repair was so fast that it was difficult to detect. We also found that the so-called 'slow' phase in both plants actually consisted of two components; an initial period of negligible repair lasting 80–120 min followed by a period of rapid repair lasting <30 min. Using *Arabidopsis* mutants homozygous for both ATM and BRCA1, we found that both of these genes are required for DNA repair during the 3-h period of our experiments, indicating that the 'slow' phase involves a homologous repair (HR) of double-strand breaks and clustered single-strand breaks. The lag of repair in the 'slow' phase presumably involves induction of expression of genes involved in HR repair such as BRCA1 and RAD51.

Introduction

Low-dose radiation exposures in the range 1–100 mGy are used extensively in medical and dental diagnostics with higher doses being used for the treatment of cancer. There continues to be interest in obtaining a better understanding of low-dose effects and in defining thresholds for detectable damage. Gaps in knowledge about low-dose radiation also affect our ability to evaluate possible environmental and public health consequences of nuclear accidents (1).

Many earlier studies on radiation focused on effects of acute exposures, including human studies looking at long-term effects at Hiroshima and Chernobyl, for example (2, 3). In fact, several of the threshold values used today for safe radiation exposure have been based on extrapolation of effects obtained with acute exposures.

Another reason why low-dose effects are inadequately understood has to do with the lack of a sufficiently sensitive method for measuring damage to DNA. In earlier studies, DNA damage was assayed using electrophoresis (4, 5) or chromatography (6). Starting 30 years ago (7, 8), the comet assay has been applied to measure DNA damage at the level of single nuclei. More recently, fluorescent antibody assays have been used for detecting foci of DNA repair in cells (9). The interpretation of what is actually being measured in both the comet and foci assays is a subject of continuing discussion (10, 11).

The assumption underlying the present research was that plants can be used as models to obtain insight into fundamental processes related to X-ray damage to DNA and repair after low-dose radiation exposures and, in particular, that *Arabidopsis* mutants can provide information on mechanisms involved in repair. Several studies have been published using the comet assay to study DNA damage and repair in plants (12–17). In addition, homozygous knock-outs of *Arabidopsis* are available for both ATM and BRCA1 genes implicated in the repair of double-strand breaks (18–20). Our objectives were to use the comet assay with rye grass plants and wild-type *Arabidopsis* plants plus homozygous *Arabidopsis* mutants for ATM and BRCA1 to define thresholds for detectable DNA damage, to study kinetics of repair and to obtain insight into mechanisms of repair after X-ray exposures.

Materials and methods

Seeds of the homologous ATM and BRCA1 mutants as well as the wild-type Columbia variety of *Arabidopsis thaliana* were germinated

in 8.5-cm-diameter plastic Petri plates containing Murashige and Skoog (21) medium, 30 g/l sucrose and 8 g/l agar under controlled environment conditions at 24°C, 16-h photoperiod and an 80 µM/m²/s light intensity. Plants at the rosette stage were used for X-ray exposure experiments. The ATM mutant was obtained from Anne Britt, University of California-Davis and the BRCA1 mutant was obtained from The European Arabidopsis Stock Centre. Rye grass (*Lolium multiflorum*) was grown in soil in a greenhouse.

Plants or isolated nuclei were irradiated with X-rays using an X-RAD 225C machine (RPS Services) set at 225 kV, 13 mA and with a 0.5-mm Cu filter. Dose rates were 0.8–1.8 Gy/min estimated with Fricke's solution at the Norwegian Institute of Public Health in Oslo, Norway.

To conduct comet assays, ~20 mg fresh weight of leaf tissue plus 0.2 ml cold phosphate-buffered saline (PBS) with 50 mM EDTA were transferred to a plastic Petri plate on ice. Leaf tissue was then sliced during 25 s under safe red light, using a scalpel to release nuclei. The suspension containing released nuclei was mixed with 0.2 ml of a solution containing 1% low melting point agarose (Sigma-Aldrich) in PBS at 37°C. A 70 µl aliquot of the nuclei/agarose mixture was then transferred to a glass slide on ice and a cover slip was placed over the suspension. After gel formation (~1 min), the cover slip was removed and the slide transferred to an electrophoresis chamber containing cold 0.3 M NaOH plus 1 mM Na_2 EDTA at 4°C in the dark. The time from the beginning of slicing to transfer of slides to the electrophoresis chamber was ~5 min. After at least 20 min for lysis and DNA unwinding, the slides were subjected to electrophoresis in the dark at 25 V, corresponding to ~300 mA for 15 min at 4°C. Afterwards, the slide was soaked in cold PBS for 10 min to neutralise pH, then dipped in 95% ethanol and dried at room temperature.

Analysis of comets was performed after staining with 1 µg/ml 4′-6-diamidino-2-phenylindole using a Nikon Eclipse TS100 fluorescence microscope equipped with an Allied Vision Technologies camera and the Comet Assay IV image analysis program (Perceptive Instruments). Fifty comets were scored per plant and the level of DNA damage was expressed as the percentage of DNA in the tail (% tail DNA) with error bars representing 95% confidence levels based on analysis of variance. As recommended (22), significance was also confirmed using the *t*-test and *P* values were calculated.

Results

Phases of DNA repair after acute exposures

Figure 1 shows data on repair after exposures of whole *Arabidopsis* plants to 5- and 15-Gy X-rays. Consistent with earlier reports with *Vicia faba* (13), DNA repair after acute 5- and 15-Gy exposures could be divided into 'rapid' and 'slow' components. After exposure of *Arabidopsis* or rye grass to 2 Gy or less, no rapid phase could be detected (Figure 2).

Comparison of DNA damage in isolated nuclei versus intact cells

The failure to demonstrate 'rapid' phases for DNA repair after X-ray exposures of whole plants corresponding to 2 Gy or less could be explained if the 'rapid' repair phases after these low exposures were completed in <10 min, i.e. within the time taken to isolate and embed nuclei. We therefore compared the damage levels after exposure of whole cells, versus isolated nuclei embedded in agarose, to a range of X-ray doses (Figure 3). DNA damage could be detected in isolated nuclei at exposures as low as 200 mGy, while a 100-mGy exposure did not cause detectable DNA damage (data not shown).

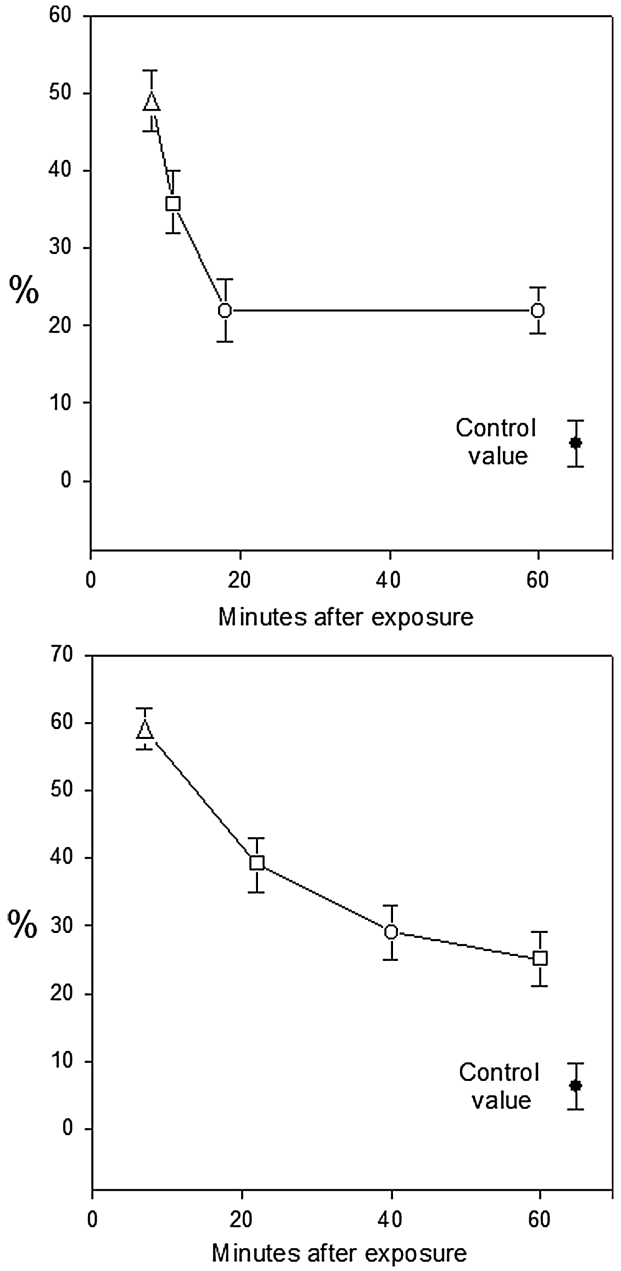

Figure 1. Demonstration of 'rapid' and 'slow' phases of DNA repair after acute X-ray exposures of *Arabidopsis*. Upper panel shows a time course of DNA repair after a 5-Gy exposure, while the lower panel shows repair after a 15-Gy exposure. Each point is the mean % tail DNA value for 50 comets with error bars representing 95% confidence levels based on analysis of variance. Different symbols (open triangles, squares and circles) indicate results obtained in different, independent experiments. Control values shown are the means of control values for the different experiments reported in the figure.

After 2 Gy (the dose applied in the experiments of Figure 2), there was significantly more DNA damage in nuclei compared to whole cells. In fact, in all cases, % tail DNA values were higher for isolated nuclei compared to the values obtained with intact cells. Asterisks (*) in Figure 3 indicate data points with *P* values <0.01 compared to controls. It seems therefore that the extra DNA damage seen in isolated nuclei compared to intact leaf cells after 2-Gy exposure represents the damage that is repaired so rapidly in whole

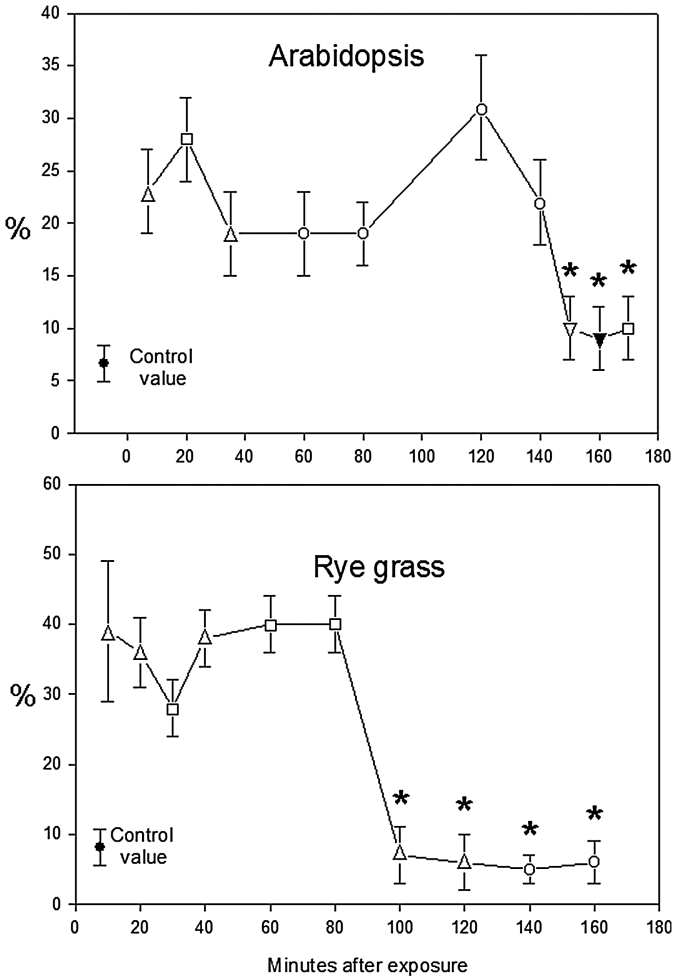

Figure 2. Time courses of DNA repair after an acute 2-Gy X-ray exposure of *Arabidopsis* (upper panel) and rye grass (lower panel). Each point represents the mean % tail DNA value for 50 comets from an individual plant with error bars showing 95% confidence levels based on analysis of variance. Data points with asterisks (*) are not significantly different from controls based on the *t*-test with *P* values >0.1. Different symbols (open triangles, squares and circles) indicate results obtained in different, independent experiments. Control values shown are the means of control values for the different experiments reported in the figure.

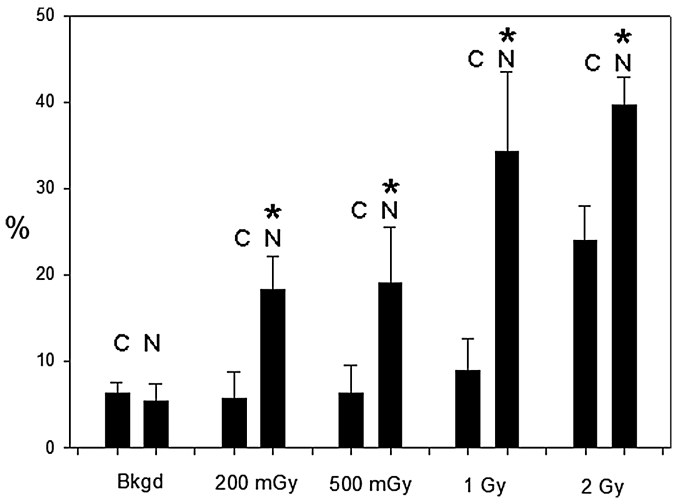

Figure 3. Dose/response for DNA damage in intact cells (C) versus isolated nuclei (N) of *Arabidopsis* exposed to X-rays. Each bar represents the mean % tail DNA value for three distinct experiments, each involving 50 comets from an individual plant, with error bars showing standard deviations. Bars for isolated nuclei with asterisks (*) are significantly greater than the corresponding bars for intact cells.

plants—within the time taken to isolate and embed nuclei—that it is undetected. Thus, after 2 Gy in the experiments of Figure 2, essentially only the slow phase was followed.

Time course of 'slow' repair

An earlier study with *V. faba* used X-ray exposures to demonstrate 'rapid' and 'slow' DNA repair phases but it failed to follow the 'slow'

phase long enough to reach control % tail DNA values (13). When we exposed rye grass and *Arabidopsis* plants to 2 Gy, we could follow the 'slow' phase for 3 h during which initial DNA damage was completely repaired (Figure 2) (data points indicated by asterisks in Figure 2 are not significantly different from controls; $P > 0.1$). It was clear in both cases that the so-called 'slow' phase consisted of two components. In *Arabidopsis*, an initial component of negligible repair during the first 2 h was followed by a fast repair component. Rye grass showed an initial component of negligible repair lasting 80 min followed by a fast repair component. It was not possible to detect intermediate stages in this fast component. The timing of the fast component is consistent with a requirement for synthesis of protein(s) following induction by the irradiation.

Priming/hormesis experiments confirm induction

We expected that it would be possible to demonstrate a priming or hormesis effect if we first exposed plants to an inductive low-dose exposure followed by an acute dose 3 h later. Figure 4 summarises different experiments related to this idea. As shown in Figures 2 and 4, a 2-Gy X-ray exposure caused significant DNA damage. By pre-exposing plants to 200 mGy followed by a 2-Gy exposure 3 h later, DNA damage was shown to be significantly lower compared to 2 Gy alone, i.e. a classical radiation hormesis effect (23). For example, the mean % tail DNA value for three independent replicates exposed to 2 Gy was 24% compared to a mean % tail DNA value of 12% for three independent replicates pre-exposed to 200 mGy and then given a 2-Gy exposure.

On the other hand, pre-exposure to 50 mGy followed by 2-Gy exposure 3 h later did not reduce DNA damage compared to 2 Gy alone, showing that the threshold for the 3 h hormesis effect lies between 50 and 200 mGy. Finally, Figure 4 shows that pre-exposure to 200 mGy followed by 2-Gy exposure 24 h later did not reduce DNA damage compared to 2 Gy alone, indicating that the induction effect is not long-lasting. The asterisk (*) in Figure 4 indicates that pre-irradiation with a 200-mGy exposure followed by a 2-Gy exposure 3 h later gave a significantly lower % tail DNA value compared with 2 Gy alone ($P < 0.01$).

Dependence on BRCA1 and ATM

To gain further insight into the nature of the 'slow' phase of DNA repair and the mechanisms involved, we conducted experiments with mutants of *Arabidopsis* homozygous for ATM and BRCA1 genes. As Figure 5 shows, neither of these mutants repaired DNA during the 3-h time course when wild-type *Arabidopsis* completely repaired damage, indicating that both ATM and BRCA1 are necessary for 'slow' phase repair.

Discussion

In agreement with the earlier investigation of DNA damage and repair after X-irradiation of *V. faba* (13), using acute exposures such as 5 or 15 Gy, we confirmed that repair could be separated into so-called 'rapid' and 'slow' phases. However, an extended examination of the 'slow' phase in both *Arabidopsis* and rye grass showed that it actually involved an initial period of negligible repair lasting 80–120 min followed by a period of rapid repair lasting <30 min.

The 'rapid' phase is so fast that methods for measuring DNA damage that involve any delay after exposure are underestimating initial levels of damage. Failure to detect damage after low doses of radiation may simply reflect this rapid repair, and so estimates of threshold doses causing no damage may be over-estimates. When the capacity for repair was reduced by irradiating *Arabidopsis* nuclei after embedding in agarose, exposure to 200 mGy was sufficient to cause detectable DNA damage.

Our results clearly show that complete repair of DNA damage after X-irradiation during the time frame of these experiments is dependent on active genes for both ATM and BRCA1. Given what is known about these genes (24–27), it seems likely that the so-called 'slow' phase of DNA repair consists of homologous repair (HR) of double-strand and clustered single-strand breaks (28, 29).

An earlier report (17) on DNA repair in *Arabidopsis* showed that repair began early and claimed that the neutral comet assay is specific for double-strand breaks, citing an earlier reference (30). However, the reference cited (30) does not support this interpretation, since the

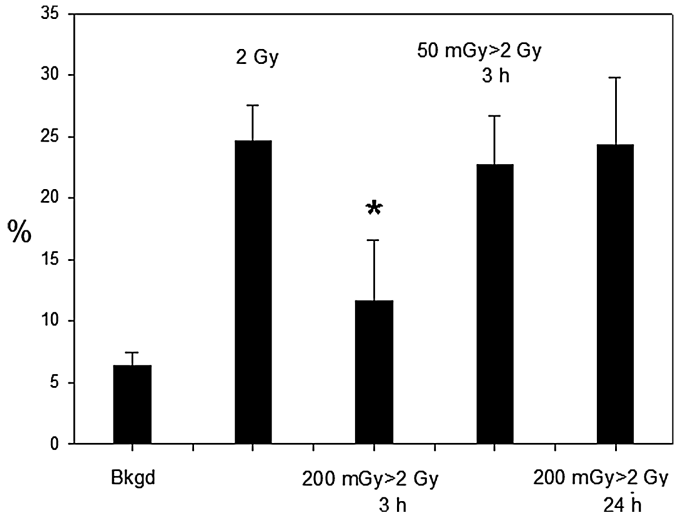

Figure 4. Low-dose exposures can protect against DNA damage caused by subsequent acute exposures. Each bar represents the mean % tail DNA value for three distinct experiments, each involving 50 comets from an individual plant, with error bars showing standard deviations. The % tail DNA values were measured 10–40 min after exposures, i.e. during the slow phase when negligible repair was detected. DNA damage is shown, from left to right, for the following: background, not irradiated; irradiated with a 2-Gy exposure; pre-irradiated with a 200-mGy exposure followed by a 2-Gy exposure 3 h later; pre-irradiated with a 50-mGy exposure followed by a 2-Gy exposure 3 h later; pre-irradiated with a 200-mGy exposure followed by a 2-Gy exposure 24 h later. The asterisk (*) indicates that pre-irradiation with a 200-mGy exposure followed by a 2-Gy exposure 3 h later gave a significantly lower % tail DNA value compared to 2 Gy alone.

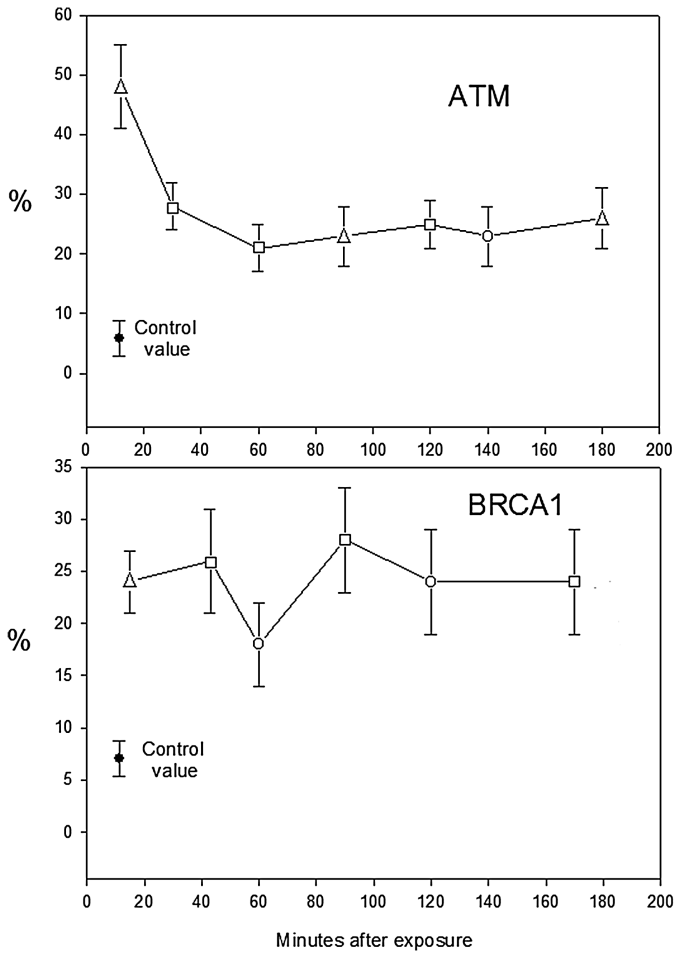

Figure 5. *Arabidopsis* ATM and BRCA1 genes are required for completed DNA repair after irradiation. Data show % tail DNA values after 2-Gy exposure of *Arabidopsis* mutants homozygous for ATM (upper panel) and BRCA1 (lower panel) genes. Each bar represents the mean % tail DNA values for 50 comets from an individual plant with error bars showing 95% confidence values based on analysis of variance. Different symbols (open triangles, squares and circles) indicate results obtained in different, independent experiments. Control values shown are the means of control values for the different experiments reported in the figure.

neutral assay was at least as sensitive at detecting damage induced by the alkylating agent methyl methanesulfonate (not known as a double strand break-inducing chemical) as were the standard alkaline assay or the alkali/neutral modifications. In other words, the neutral assay can detect single-strand breaks.

So, the explanation for delayed rapid repair during the 'slow' phase probably involves the induction of enzymes involved in HR, including BRCA1 and RAD51 which are both strongly up-regulated by X-rays in *Arabidopsis* (18, 20, 31). Up-regulation probably also explains the priming/hormesis effects we observed. A pre-exposure to 200 mGy protected cells from subsequent damage 3 h later, but 50-mGy pre-exposures were ineffective at protection, showing that induction has a threshold in the range 50–200 mGy. Likewise, 200-mGy exposures followed by acute exposures 24 h later did not cause protection, indicating that the induction of the HR is relatively short term.

The question can be asked how G1 leaf cells perform HR without replicated copies of individual chromosomes. As shown in yeast, G1 cells form HR foci after DNA damage (32).

With regard to plants, it has been demonstrated that HR repair can occur in leaf cells of *Arabidopsis* by a mechanism involving homology between repeated sequences in the region of DNA damage, an event that results in excision of intervening sequences and recombination events (33–35).

Conflict of interest statement: None declared.

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