Evidence of an Adaptive Response Targeting DNA Nonhomologous End Joining and Its Transmission to Bystander Cells

Holger Klammer, Munira Kadhim, and George Iliakis

Abstract

Adaptive response (AR) is a term describing resistance to ionizing radiation–induced killing or formation of aberrant chromosomes that is mediated by pre-exposure to low ionizing radiation doses. The mechanism of AR remains elusive. Because cell killing and chromosome aberration formation derive from erroneous processing of DNA double-strand breaks (DSB), AR may reflect a modulation of DSB processing by nonhomologous end joining (NHEJ) or homologous recombination repair (6–9). In NHEJ, the ends of the broken DNA molecule are processed and ligated in a manner that does not require homology and which may alter the DNA sequence near the DSB. In its classic form, NHEJ requires the activities of KU and DNA-PKcs, and ligation is mediated by the DNA ligase IV-XRCC4-XLF complex (8, 10). We term this form D-NHEJ to indicate its dependence on DNA-PKcs (10, 11). In addition to D-NHEJ, several reports indicate the function of an alternative pathway of end joining that is normally suppressed by D-NHEJ and which functions as a backup (B-NHEJ; refs. 10–12). There is evidence that the DNA ligase III/PARP1/XRCC1 complex normally involved in the repair of DNA single-strand breaks also functions in B-NHEJ. In contrast to NHEJ, repair of a DSB by homologous recombination repair requires a region of extensive homology that is normally suppressed by D-NHEJ and which functions as a backup (B-NHEJ; refs. 10–12). There is evidence that the DNA ligase III/PARP1/XRCC1 complex normally involved in the repair of DNA single-strand breaks also functions in B-NHEJ. In contrast to NHEJ, repair of a DSB by homologous recombination repair requires a region of extensive homology that does not require homology and which may alter the DNA sequence near the DSB. In its classic form, NHEJ requires the activities of KU and DNA-PKcs, and ligation is mediated by the DNA ligase IV-XRCC4-XLF complex (8, 10). We term this form D-NHEJ to indicate its dependence on DNA-PKcs (10, 11). In addition to D-NHEJ, several reports indicate the function of an alternative pathway of end joining that is normally suppressed by D-NHEJ and which functions as a backup (B-NHEJ; refs. 10–12). There is evidence that the DNA ligase III/PARP1/XRCC1 complex normally involved in the repair of DNA single-strand breaks also functions in B-NHEJ. In contrast to NHEJ, repair of a DSB by homologous recombination repair requires a region of extensive homology that is normally suppressed by D-NHEJ and which functions as a backup (B-NHEJ; refs. 10–12). There is evidence that the DNA ligase III/PARP1/XRCC1 complex normally involved in the repair of DNA single-strand breaks also functions in B-NHEJ. In contrast to NHEJ, repair of a DSB by homologous recombination repair requires a region of extensive homology that is normally suppressed by D-NHEJ and which functions as a backup (B-NHEJ; refs. 10–12). There is evidence that the DNA ligase III/PARP1/XRCC1 complex normally involved in the repair of DNA single-strand breaks also functions in B-NHEJ. In contrast to NHEJ, repair of a DSB by homologous recombination repair requires a region of extensive homology that is normally suppressed by D-NHEJ and which functions as a backup (B-NHEJ; refs. 10–12). There is evidence that the DNA ligase III/PARP1/XRCC1 complex normally involved in the repair of DNA single-strand breaks also functions in B-NHEJ. In contrast to NHEJ, repair of a DSB by homologous recombination repair requires a region of extensive homology that is normally suppressed by D-NHEJ and which functions as a backup (B-NHEJ; refs. 10–12). There is evidence that the DNA ligase III/PARP1/XRCC1 complex normally involved in the repair of DNA single-strand breaks also functions in B-NHEJ. In contrast to NHEJ, repair of a DSB by homologous recombination repair requires a region of extensive homology that is normally suppressed by D-NHEJ and which functions as a backup (B-NHEJ; refs. 10–12). There is evidence that the DNA ligase III/PARP1/XRCC1 complex normally involved in the repair of DNA single-strand breaks also functions in B-NHEJ. In contrast to NHEJ, repair of a DSB by homologous recombination repair requires a region of extensive homology that...
in the late S and G2 phases of the cell cycle (13, 14). Whether any of these DSB repair pathways are upregulated by the development of AR has not been investigated, although the question remains central for a mechanistic understanding of the effect.

In the present study, we test the hypothesis that upregulation of D-NHEJ under conditions supporting the development of AR is detectable by in vivo plasmid end-joining assays. These assays offer means for assaying NHEJ activity on defined DSBs introduced by restriction endonucleases in a known DNA context. These major advantages leverage the fact that simple DSBs introduced by restriction endonuclease in extrachromosomal substrates model only certain aspects of ionizing radiation–induced complex genomic DSBs.

We show robust increases in NHEJ efficiency in mouse embryonic fibroblasts and human cells pre-exposed to low doses of ionizing radiation, irrespective of reporter plasmid used, and show that they are sensitive to inhibitors of DNA damage signaling. Growth medium of pre-irradiated cells induces an AR in nonirradiated bystander cells similar in magnitude to that seen in irradiated cells. The results point to enhanced NHEJ as a mechanism of AR and suggest that AR may be transmitted to bystander cells through factors generating replication-mediated DSBs.

**Materials and Methods**

**Cell lines and irradiation**

Mouse embryonic fibroblasts from p53+/− and p53+/−LIG4−/− animals (a gift of Dr. Fred Alt, Department of Genetics, Harvard Medical School, Boston, MA) were maintained in DMEM and human A549 lung epithelial carcinoma cells (American Type Culture Collection) in McCoy's 5A medium, both supplemented with 10% fetal bovine serum (PAA Laboratories), penicillin, and streptomycin. All cell lines tested negative for mycoplasma and their genotype confirmed in our laboratory by PCR.

Cells were irradiated with the use of an X-ray machine (GE Healthcare) operated at 320 kV, 10 mA with a 1.6-mm Al filter. The dose rate at 50 cm was 2.7 Gy/min.

**In vivo end-joining plasmid assays**

Standard experimental protocols for AR include exposure of cells to a conditioning dose, a period of incubation for adaptation, and an exposure to a challenging dose, in which the cellular response is measured with the use of a specific endpoint. Here we exposed cells to a conditioning ionizing radiation dose and incubated them for adaptation. Instead of a challenging dose we introduced a plasmid containing a restriction endonuclease–induced DSB, the repair of which was quantitatively evaluated through signals generated by appropriately selected reporter genes. The pEGFP-Pem1-Ad2 plasmid (15) was used for this purpose, as described previously (11). It carries the reporter gene EGFP engineered with the Pem1 intron that is here interrupted by the adenoviral exon 2, Ad2 (Fig. 1A). Because Ad2 disrupts EGFP, this form of the plasmid does not express functional EGFP. Digestion at both the upstream-engineered and downstream-engineered HindIII sites removes the exon and generates a DSB with compatible 5′ overhangs. Successful in vivo rejoining of this DSB allows EGFP expression that can be quantified by flow cytometry. For calibration purposes the pEGFP-Pem1 form of the plasmid lacking the Ad2 exon was used.

Circular pEGFP-Pem1 or HindIII linearized pEGFP-Pem1-Ad2 were cotransfected with the pDsRed2-N1 plasmid (Clontech). The resulting red signal is measured in parallel with the green EGFP signal and is used to normalize for transfection efficiency. The NHEJ efficiency is estimated based on the calibration curves measured in parallel. It is shown as the percentage of the transfected plasmid that is rejoined.

The pCMS-end plasmid (Fig. 2A) has been previously described (16). It carries the reporter gene EYFP that can be separated from its CMV-P promoter by a DSB introduced by XhoI in an upstream multiclonic site. NHEJ is monitored by the restoration of the EYFP signal. The EGFP gene present in the same plasmid is used to monitor transfection efficiency. For quantification, calibration curves are generated with circular pCMS-end (Fig. 2B). The internal calibration with EGFP in this plasmid is elegant and obviates the need for cotransfection with a second plasmid. It assumes, however, that circular and linearized plasmids express EGFP with similar efficiency.

Transfections were carried out with the Amaxa Nucleofector system with the use of the MEF1 kit. Expression of EYFP (yellow) and EGFP (green), or EGFP (green) and DsRed (red) were simultaneously measured by flow cytometry for the pCMS-end and the pEGFP-Pem1-Ad2 system, respectively. A Beckman Coulter Epics XL-MCL flow cytometer equipped with an argon laser emitting at 488 nm was used. Yellow fluorescence was measured at 550 nm, green fluorescence at 510 nm, and red fluorescence at 610 nm. Compensation settings for simultaneous detection of EGFP and DsRed were 10% and 3%, and for simultaneous detection of EYFP and EGFP were 65.5% and 44%, respectively.

**Immunofluorescence microscopy**

For the immunofluorescence analysis of γH2AX and Rad51 foci, cells were grown on coverslips. After irradiation, cells were incubated for 1 hour to allow foci development to reach a maximum (established in preliminary experiments). Samples were rinsed with PBS, fixed for 15 minutes in 2% paraformaldehyde, and rinsed again. Cells were permeabilized for 10 minutes in a 0.5% Triton X-100 solution in 100 mmol/L Tris and 50 mmol/L EDTA, and rinsed with PBS followed by overnight incubation for blocking at 4°C in a PBS solution containing 0.2% gelatin and 0.5% bovine serum albumin fraction V.

For antibody staining, samples were incubated with a 1:200 solution of a monoclonal mouse antibody raised against either γH2AX (clone 3F2, Abcam) or Rad51 (14B4, GeneTex) for 1.5 hours and extensively washed with PBS. Samples were incubated for 1 hour in a 1:400 solution of the anti-mouse Alexa 488–conjugated immunoglobulin...
(Invitrogen), washed thoroughly, and counterstained for 15 minutes in 2 μg/ml 4,6-diamidino-2-phenylindole (DAPI) solution in 0.1 mol/L Tris, 0.1 mol/L NaCl, 5 mmol/L MgCl₂, and 0.05% Triton X-100. After washing samples with PBS, coverslips were mounted with ProLong Gold Antifade (Invitrogen). Samples were scanned at ×40 in an automated analysis station equipped with a fluorescence microscope (Axio Imager Z2, Zeiss) and controlled by the Metafer software (MetaSystems). The DAPI signal intensity was used to distinguish and analyze cells separately early in the cell cycle (i.e., G₁ and early S, termed here as G₁) and late in the cell cycle (i.e., late S and G₂, termed here as G₂). On average, 2,500 G₁ and 800 G₂ cells were scored per sample.

Results

A plasmid assay detects AR targeting D-NHEJ

The pEGFP-Pem1-Ad2 plasmid has been extensively used to analyze key aspects of NHEJ in cells of different genetic backgrounds (15, 17, 18). Complete digestion of this plasmid with the use of HindIII removes the disrupting Ad2 exon and generates a DSB (Fig. 1A). Expression of EGFP from the linearized plasmid is possible upon transfection only if repair of the DSB restores the intron. Only NHEJ can process this DSB as a result of the plasmid design and the lack of homologous EGFP sequences in the mammalian genome. The efficiency of NHEJ in this assay is...
quantitatively estimated by the proportion of EGFP-positive cells, which is a measure of the degree of circularization by the NHEJ apparatus of the transfected cell. Indeed, the calibration curve in Fig. 1B shows an increase in EGFP-positive cells with increasing amount of circular pEGFP-Pem1 plasmid (used here because it lacks Ad2 and expresses EGFP in its circular form). The nonlinear relationship between plasmid amount and fraction of EGFP-positive cells made the generation of such calibration curves in each experiment necessary to obtain quantitative estimates of repair efficiency. The normalization of the EGFP signal to the DsRed signal from the cotransfected control plasmid provides an internal transfection control.

In the cell, plasmid rejoining can be mediated either by D-NHEJ or by B-NHEJ. To estimate the relative contribution of each pathway, we analyzed repair proficient and D-NHEJ-deficient LIG4-/- MEFs. The results obtained are summarized in Fig. 1C. A 10-fold decrease in repair capacity is observed in LIG4-/- as compared with wild-type mouse embryo fibroblasts, suggesting that in repair-proficient cells this plasmid assay measures predominantly the function of D-NHEJ. This conclusion could also be confirmed in DNA-PKcs-deficient and KU80-deficient mutants (data not shown).

We used this assay to examine whether low doses of ionizing radiation modulate D-NHEJ. Wild-type mouse embryo
fibroblasts were exposed to 10 to 1,000 mGy and were incubated for 2 hours. Subsequently, cells were transfected with 50 ng of HindIII-linearized pEGFP-Pem1-Ad2 plasmid and incubated for 24 hours at 37°C to allow EGFP expression. The repair efficiency of the different populations is shown in Fig. 1D, normalized to that measured in sham-irradiated samples. A robust increase in NHEJ is observed above 30 mGy, reaching a maximum at 100 mGy. This statistically significant increase in NHEJ efficiency is equivalent to the development of an AR by enhancing D-NHEJ. Figure 1E shows the kinetics of development of this AR in cells exposed to 100 mGy and tested at different times thereafter. A fluctuating pattern of AR development is observed that reaches a peak at ~2 hours, a valley at 4 to 6 hours, and a second increase at later times.

To rule out that the AR measured above for mouse embryo fibroblasts was species-specific, we carried out experiments with the use of the human A549 cell line. Cells were exposed to 50 or 100 mGy and transfected with the HindIII-linearized pEGFP-Pem1-Ad2 plasmid 2 hours later. The results of EGFP expression summarized in Fig. 1F document a robust AR of a magnitude similar to that measured in mouse embryo fibroblasts.

To examine whether the development of AR relies on DNA damage signaling, we carried out experiments with caffeine, an inhibitor of the phosphatidylinositol kinase family of protein kinases that are functionally located at the apex of this signaling cascade. Cells were handled as above, but caffeine was added 1 hour before and maintained 2 hours after exposure to ionizing radiation. Notably, caffeine completely abrogates AR, suggesting that its development is mechanistically linked to the activation of caffeine-sensitive kinases.

To rule out that the above results of AR development are plasmid-specific, we carried out experiments with the use of the pCMS-end plasmid (Fig. 2A). In this plasmid, a CMV promoter drives EYFP, and this interaction can be disrupted by a DSB introduced in the multicloning site by XhoI digestion. This plasmid also expresses EGFP from a SV40 promoter that remains unaffected by this digestion and can be used as transfection control. Figure 2B shows a typical calibration curve, and Fig. 2C shows that this plasmid assay also measures mainly D-NHEJ in repair-proficient cells. Figure 2D shows evidence for AR in mouse embryo fibroblasts exposed to 10 to 1,000 mGy. Here again, a maximum is reached at 100 mGy, although the effect does not reach statistical significance in the pooled set of experiments (see Discussion). Individual experiments, on the other hand, show a robust response as indicated by the kinetics experiment summarized in Fig. 2E. As with pEGFP-Pem1-Ad2, here again a peak is reached at 2 to 4 hours, followed by a minimum and a subsequent increase.

**Adaptive response can be transmitted to nonirradiated bystander cells**

We inquired whether the above-documented AR can be transmitted to nonirradiated bystander cells. For this purpose, we exposed mouse embryo fibroblasts to ionizing radiation and transferred their medium 2 hours later to nonirradiated replicate cultures prepared in parallel and treated identically. D-NHEJ was measured in these nonirradiated cultures by transfecting the pEGFP-Pem1-Ad2 plasmid 2 hours after medium transfer. Figure 3A summarizes the results obtained and indicates a robust AR of magnitude and dose response similar to that observed in directly irradiated cells. This result was qualitatively reproduced in an experiment carried out with the pCMS-end plasmid (data not shown). The kinetics of development of this response through medium transfer (Fig. 3B) closely follows the kinetics of AR shown in Fig. 1E.

The development of AR is also detected in bystander experiments carried out with A549 cells (Fig. 3C). Nonirradiated bystander cells exposed to medium of pre-irradiated replicate cultures show a measurable AR that reaches significance at 500 mGy. In preliminary experiments, AR was seen at doses as low as 100 mGy, but these results were inconsistent. Notably, the abrogation of this effect after treatment exclusively of the bystander cells with 4 mmol/L caffeine again implicates the phosphatidylinositol kinase family of protein kinases in the response developed in bystander cells.

**DNA damage is induced in bystander cells**

The AR measured in bystander cells could in principle be induced by the generation and transmission from the irradiated cells of a factor(s) that modifies cellular physiology and enhances the repair capacity without directly generating DNA damage. Alternatively, it is possible that irradiated cells release compounds into the medium that enter bystander cells and generate DNA damage that subsequently induces AR in a manner that may be similar to that seen in irradiated cells. Because the effect of caffeine hints at the production of DNA damage—activating phosphatidylinositol kinase family of protein kinases, we examined the presence of DSBs in bystander cells by analyzing γH2AX foci formation. The automated analysis system used in these experiments offered excellent statistics through the scoring of very large numbers of cells and allowed the independent analysis of cells early and late in the cell cycle (G1 and G2 cells in our terminology) by using the DAPI signal. A549 cells were exposed to doses between 0 to 1,000 mGy and their medium transferred to nonirradiated replicates 2 hours later. γH2AX were scored in the latter cells 1 hour after medium transfer. Figure 4A shows no significant increase in foci numbers in G1 cells, which precludes the direct induction of DSBs. On the other hand, a small reproducible increase in foci number is observed in G2 cells at doses above 100 mGy (Fig. 4B), suggesting that the medium of irradiated cells contains factors generating DSBs specifically in cells in the latter part of the cell cycle.

The presence of DSBs in bystander cells specifically in the latter part of the cell cycle suggested that they may be replication-dependent. Because replication-dependent DSBs are one-sided, they preferentially use homologous recombination for their repair. To examine this possibility we analyzed Rad51 foci formation as a marker for ongoing homologous recombination repair. As expected, no Rad51 foci formed in bystander G1 A549 cells (Fig. 4C). On the other hand, an increase in Rad51 foci was observed in G2 cells, of a magnitude
comparable (within the experimental uncertainties) with that of γH2AX foci (Fig. 4D). Thus, AR in bystander cells may be induced by replication-dependent DSBs repaired by homologous recombination repair and which are induced by factors released into the medium from irradiated cells. It is interesting that although these DSBs are predominantly repaired by homologous recombination repair, they upregulate the capacity of the cells to carry out D-NHEJ.

Discussion

**In vivo plasmid end-joining assays detect adaptive response**

The results of plasmid end-joining assays presented above provide tantalizing evidence for the development of AR in various types of cells. The effect size of 25% is robust and matches other reports of AR induction showing values between 6% (19) and 59% (20). An effect is detectable at 100 mGy, and an increase is observed up to ∼1,000 mGy. This dose range of AR induction also matches other published results (21). The typical model for AR assumes optimal induction at a given radiation dose and lower levels of induction at doses lower or higher than this optimal dose (22). In our experiments, a drop in the level of AR is not evident for doses up to 1 Gy.

The results reported here are corroborated by two independent assays. However, strong fluctuations were partly observed between different experiments, and these are the reasons why the increase in D-NHEJ as a function of dose did not reach statistical significance with the second plasmid. However, taken individually, the majority of experiments showed a robust effect. Fluctuations in the level of AR induction are not specific to our experimental conditions and have been reported for other systems as well (23). Sources of irreproducibility include the growth conditions, handling, and fluctuations in the distribution of cells throughout the cell cycle.

Also, plasmid design may contribute to the magnitude of the fluctuations seen. Thus, the pCMS-end plasmid is linearized in the multiclonic site situated between the promoter and the EYFP reporter. Small deletions or nucleotide additions during repair may generate a plasmid unable to express EYFP. In contrast, the 1.5-kb Pem1 intron of pEGFP-Pem1-Ad2 can buffer large deletions without an effect on EGFP expression. Finally, the internal calibration through EGFP of pCMS-end assumes equally efficient expression from a linear or a circular plasmid. However, especially designed experiments show that linear pCMS-end generates ∼30% lower signal than an equal amount of circular plasmid. This effect may have contributed to the larger AR fluctuations observed with this plasmid.

AR development is detectable 1 to 4 hours after exposure to ionizing radiation. This is in line with several other reports in the literature that use *in vitro* systems (23). However, *in vivo* models tend to show slower kinetics of AR development and typically require days or even months (21). We observed a rather complex pattern of AR development, which in its salient features was similar for both assays.

Figure 3. AR can be induced in nonirradiated bystander cells by transferring medium from irradiated cells. A, D-NHEJ efficiency in nonirradiated MEFs incubated for 2 hours in medium obtained from parallel cultures exposed to the indicated doses and incubated at 37°C for 2 hours. An increase in D-NHEJ is observed above 30 mGy, with a calculated value of *P* < 0.05 by ANOVA. The mean and SE from three experiments are shown. B, D-NHEJ in nonirradiated MEFs that received medium from MEFs exposed to 100 mGy and incubated at 37°C for the indicated times. The results are from a single representative experiment. Qualitatively similar results were obtained in additional experiments. C, D-NHEJ efficiency in nonirradiated A549 incubated for 2 hours in medium obtained from parallel cultures exposed to the indicated doses and incubated at 37°C for 2 hours. The inhibitor caffeine was added before medium transfer at a concentration of 4 mmol/L. Shown are the mean and SE from two to four experiments.
The sources of these fluctuations are not understood, but they may reflect, at least partly, the progression of cells through the cell cycle or the activation chain of DNA damage signaling.

Enhancement of D-NHEJ as mechanism of AR

Perhaps the most important conclusion of the work presented here is that development of AR is associated with an enhancement in the efficiency of D-NHEJ. A recent article arrives at similar conclusions with the use of a different experimental approach (24). In this work, functional D-NHEJ was shown to be required for the development of AR, measured as increase in survival after a challenging dose that is given after a small conditioning dose. Whereas in our work the experimental protocols used could not detect with statistical significance the AR at the cell survival level (results not shown), increased survival is a frequently used end point in such experiments. Modulations in cell radiosensitivity to killing are likely to have important ramifications in the clinical setting, in which tumors are treated with multiple fractions of radiation given over several weeks. Therefore, mechanistic information on the mechanisms underlying AR may provide a rationale for the further improvement of radiation therapy.

In addition to the involvement of D-NHEJ in the development of AR, our results also implicate caffeine-sensitive kinases, such as ATM, ATR, and DNA-PKcs, in the overall response. Probably, the observed enhancement in D-NHEJ is mediated by the activation of the DNA damage–signaling cascade that induces activating post-translational modification in participating proteins. Indeed, it has been reported that low radiation doses enhance the activity of DNA-PKcs (24). Also, connections between D-NHEJ and other signaling cascades, such as the EGFR growth signaling cascade, have been established (25), and similar connections may also regulate B-NHEJ (26). Finally, in some systems AR is mediated by activation of nuclear factor-κB (NF-κB; ref. 27), a downstream target of several signal transduction pathways, and evidence for cooperation between ATM, ERK, and NF-κB in the induction of AR has been reported (28).

Whereas our experiments focus on D-NHEJ as a contributor to AR, other experiments implicate other repair pathways as well. Thus, with the use of a set of knockout Chinese hamster ovary cell lines, the involvement of nucleotide excision repair has been suggested (29). Indeed, nucleotide excision repair is upregulated after exposure to low radiation doses (30–32). Whereas a contribution of nucleotide excision repair to AR is likely, its relative contribution may be smaller than that of D-NHEJ. Also, the question arises about whether B-NHEJ and possibly also homologous recombination repair somehow contribute to AR. Further experiments and robust AR assays will be necessary to address these highly relevant issues.

Figure 4. Bystander cells exposed to medium of irradiated cells develop replication-dependent DSBs. Background corrected γH2AX and Rad51 foci numbers in nonirradiated A549 cells incubated for 1 hour in medium obtained from parallel cultures exposed to the indicated doses and incubated at 37°C for 2 hours. A, γH2AX foci in G1 cells. B, γH2AX foci in G2 cells. C, Rad51 foci in G1 cells. D, Rad51 foci in G2 cells. The mean and SE from four experiments are shown.
AR is induced by media transfer in bystander cells

The observation that medium from irradiated cells can upregulate D-NHEJ at a magnitude similar to that measured with directly irradiated cells suggests that AR responses can be transmitted to bystander cells. The range of doses in which this effect is observed is similar to that typically used in bystander experiments (33–36). Also, the kinetics of the effect is similar to that measured in bystander experiments with the use of medium transfer protocols (37).

It is relevant to point out that the bystander response documented here is of beneficial nature. Although such beneficial bystander responses have been reported (38), bystander effects are typically associated with the transmission of adverse radiation consequences to nonirradiated cells. Notably, there is evidence that detrimental and beneficial bystander responses are mutually exclusive (39–41). But what sort of factors in the medium of irradiated cells induces AR in nonirradiated bystander cells? Whereas AR measured in bystander cells could in principle be induced by factors modifying cellular physiology without directly generating DNA damage, the results presented show that damage induction in bystander cells actually underlies the observed response. Factors present in the medium of irradiated cells somehow collaborate with DNA replication and generate DSBs. This is why damage is only observed in cells in the later part of the cell cycle that includes S and G2 cells. These DSBs are likely to be one-sided and specifically removed by homologous recombination repair, which explains the similarity in the numbers of γH2AX and Rad51 foci observed. Thus, similarly to directly irradiated cells, bystander cells develop adaptive response by “seeing” DNA damage. This also explains why caffeine abrogates the response. Development of γH2AX foci, specifically in S-phase cells, after transfer of medium from irradiated cultures was previously reported in 4BR human fibroblasts and the T98G human glioma cells (40). The identity of factors mediating this response remains elusive, but they may act by generating oxidative stress (42).

In summary, the results presented here point to enhanced D-NHEJ as a mechanism of AR and suggest that AR may be transmitted to bystander cells through factors generating replication-mediated DSBs. Further studies will be required to elucidate the mechanism of D-NHEJ enhancement and identify the factors responsible for DNA replication–mediated DSBs. The importance of AR in radiation protection and radiation therapy makes such efforts worthwhile.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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