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Cellular Response to Pulsed Low-dose Rate Irradiation in X-ray Sensitive Hamster Mutant Cell Lines

N. CASTRO KREDER, R. ten CATE, H. M. RODERMOND, C. van BREE, N. A. P. FRANKEN, M. Z. ZDZIENICKA, and J. HAVEMAN*

HDR irradiation/LDR irradiation/Dose rate effect/DNA repair/Hamster mutant cell lines

The role of DNA repair mechanisms in the cellular response to low dose rate (LDR) irradiation was studied with the aim to gain insight in the process of sublethal damage (SLD) repair. Chinese hamster cell lines mutated in either DNA single strand break (ssb) repair or DNA double strand break (dsb) repair by non homologous end joining (NHEJ) and homologous recombination (HR), or showing an AT-like phenotype, were irradiated in plateau-phase either at high dose rate (HDR, 3.3 Gy/min) or at pulsed low dose rate (p-LDR, average 1 Gy/h). Cell survival after irradiation was assessed using the clonogenic assay. A change in sensitivity when the dose rate was decreased was observed for all parental cell lines and the DNA ssb repair mutant. No difference in cell survival after p-LDR versus HDR irradiation was observed for the two NHEJ mutants, the AT-like mutant and the HR mutant. Based on these results we conclude that single strand break repair does not play a role in the dose rate effect. The AT like protein, functional NHEJ and XRCC3 are required for the dose rate effect.

INTRODUCTION

The dose rate with which radiation is delivered significantly affects the biological response to radiation, especially in sparsely ionizing types of radiation such as X-rays or γ-rays. Reducing the dose rate decreases the biological effect. This implies that when the dose rate is reduced, to obtain the same biological effect we must administer a higher dose.

Most studies on the biological effects of LDR were performed in the 1960s until the 1980s. In this period, Bedford and collaborators showed the effect of continuous low-dose rate irradiation on a range of mammalian cells, and they demonstrated the importance of cell proliferation, cell cycle redistribution, and damage repair on the observed effect. We now know that as the radiation dose rate is reduced, the irradiation time increases, allowing processes like repair, cell cycle redistribution, and repopulation to take place during irradiation. These processes give rise to the so-called dose-rate effect, which refers to a change in sensitivity when the dose rate is modified. Conventional radiation schemes use high-dose rate (HDR) irradiation, which takes only minutes to complete, and thus the radiation time is too short for these processes to take place.

Ionizing radiation causes numerous types of DNA damage, including single- and double-strand breaks (ssb and dsb, respectively), base damage, and DNA-protein cross links. This DNA damage is repaired highly efficiently by several distinct DNA repair mechanisms. Among the different types of lesions, the DNA dsb is considered by far the most lethal. If left unrepaired, it causes cell death in the first mitosis, and when it is repaired incorrectly, it gives rise to chromosomal aberrations that can ultimately lead to malignant transformation. There are two major pathways by which dsb’s can be repaired, nonhomologous end joining (NHEJ) and homologous recombination (HR).

The use of rodent mutant X-ray-sensitive cell lines has greatly enhanced the knowledge of the cellular response to radiation. Eleven complementation groups have been established identifying genes involved in the radiation response. Of these groups, one shows defective ssb repair; three others are involved in homologous recombination (XRCC2, XRCC3, XRCC11); four groups show reduced DNA dsb repair and were identified to be a part of NHEJ (XRCC4, XRCC5, XRCC6, XRCC7); and one group shows AT-like characteristics (XRCC8). DNA repair plays a major role in the dose-rate effect for...
irradiations that are significantly longer than 15 min\(^9\). The type of damage repair responsible for the dose-rate effect is the repair of sublethal damage (SLD)\(^{22}\). SLD is damage that can be repaired or accumulated with further dose to become lethal.\(^{23}\) It is repaired during irradiation or between fractions of low doses of HDR.

More and more becomes known about the role of the different DNA repair mechanisms in response to ionizing radiation. However, most of these data is obtained after HDR irradiation. To study DNA repair after low-dose rate (LDR) irradiation, we used a panel of Chinese hamster cell lines that are deficient in ssb repair, NHEJ, HR, or they show an AT-like phenotype, and we compared their behavior after HDR and LDR irradiation. Plateau-phase cell cultures were used to minimize the effects of repopulation and cell cycle redistribution.

**MATERIALS AND METHODS**

**Cell lines**

In this study, five mutant hamster cell lines with the corresponding parental cell lines were used. The characteristics of the cell lines are summarized in Table 1. All cell lines were cultured in F10 medium supplemented with 10% fetal calf serum, 5 mM glutamine, and 50 \(\mu\)g/ml gentamicin and in 5% CO\(_2\) at 37°C in an incubator.

The confluent “plateau phase” cultures were obtained by plating appropriate numbers of cells into 35 mm culture dishes. Confluent cultures were obtained usually in 2–3 days. For both the HDR and p-LDR experiments all cell lines were irradiated in the plateau phase. Furthermore, the irs-1SF cell line was irradiated in the exponentially growing phase.

**Irradiation**

HDR irradiation and pulsed LDR (p-LDR) irradiations were performed with a Siemens Stabiliplan 2 X-ray machine (Siemens, Germany). HDR irradiation was performed at 3.3 Gy/min. The distance between the focus and the culture dish was 33.5 cm, and a 0.5 mm Cu filter was used.

For p-LDR, irradiation, the pulse dose was 0.1 Gy at 9.16 cGy/min, the resting period between the pulses was 4 min 52 s, and this resulted in a mean dose rate of 1 Gy/h. The distance between the focus and the culture dish was 1.60 m, and a 1 mm Cu filter was used. During irradiation, the cells were kept at 37°C in a water bath. A maximum of six 100 mm culture dishes, fifteen 60 mm culture dishes, or thirty-six 35 mm culture dishes could be irradiated simultaneously with a 95% dose homogeneity. Dose output was inspected once a month with a BF-vat detector and a Farmer electrometer.

**Flowcytometric analysis of cell cycle distribution**

The cells were plated and treated as described above. At the time of radiation, 10 \(\mu\)M bromodeoxyuridine (BrdU) was administered from a 100x stock. After 2 hours the cells were harvested, fixed in 70% ethanol in phosphate buffered saline (PBS), and stored at –20°C until immunofluorescent staining. Ethanol-fixed cells were centrifuged (1 min, 2,200 rpm), resuspended in 1 ml pepsin solution (0.4 mg/ml 0.1N HCl), and incubated for 30 min at room temperature. Subsequently, the DNA was denatured by a 30 min incubation in 1 ml 2N HCl at 37°C. After washing with PBTb (PBS, Tween-20 0.05% v/v, bovine serum albumin (Sigma) 20 mg/ml, pH 7.4, the pellet was resuspended in 0.1 ml rat anti-BrdU (Harlan Seralab Ltd., Loughborough, UK, diluted 1:100 in PBTb) and incubated at room temperature for 30 min. After being washed with PBTg (PBS, Tween-20 0.005 v/v, normal goat serum [Dako, Glostrup, Denmark] 1% v/v, pH 7.4), the pellet was resuspended in 0.1 fluorescein conjugated goat-anti-rat IgG (Jackson, nr 112-015-102, West Grove, Pennsylvania, USA, diluted 1:100 in PBTg) and incubated at room temperature in the dark for 30 min. Pro-pidium-iodine and ethanol were added to an end-concentration of 1 \(\mu\)g/ml and 30%, respectively. Samples were stored at 4°C until flowcytometric analysis. They were syringed through a 21 gauge needle to reduce cell aggregation before flowcytometry (FACScan cytometer, Becton Dickenson, San Jose, CA). The distribution of cells over the cell cycle was analyzed with Windows Multiple Document Interface Flow Cytometry Application (WinMDI) by placing windows around G0/G1, S, and G2/M populations.

**Clonogenic Assay**

Cell survival was determined by clonogenic assay. In brief, at 24 hours after HRD irradiation or directly after p-

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Parent</th>
<th>Description</th>
<th>Isolated by (ref)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM-C11</td>
<td>CHO9</td>
<td>XRCC1, defective ssb repair</td>
<td>Zdzienicka et al.(^{24})</td>
</tr>
<tr>
<td>irs1SF</td>
<td>AA8</td>
<td>XRCC3, Rad51 family, recombinational repair</td>
<td>Fuller and Painter(^{25})</td>
</tr>
<tr>
<td>XR-V15B</td>
<td>V79B</td>
<td>XRCC5, Ku80 mutant, defective dsb repair (NHEJ)</td>
<td>Zdzienicka et al.(^{26})</td>
</tr>
<tr>
<td>XR-C1</td>
<td>CHO9</td>
<td>XRCC7, DNA-PKcs mutant, defective dsb repair (NHEJ)</td>
<td>Errami et al.(^{27})</td>
</tr>
<tr>
<td>V-C4</td>
<td>V79</td>
<td>XRCC8, AT-like mutant</td>
<td>Zdzienicka et al.(^{28})</td>
</tr>
</tbody>
</table>
LDR irradiation, the cells were trypsinized and replated in appropriate dilutions in 6-well culture plates (Costar). Eight days later the colonies were fixed in 6% glutaraldehyde and stained with 0.05% crystal violet. Colonies of 50 cells or more were scored as originating from a single clonogenic cell. Plating efficiencies for parental and mutant cells were as follows (mean, percent, ± standard deviation from at least three experiments): CHO9 98 ± 7, EM-C11 94 ± 15, AA8 98 ± 16, irst1SF 43 ± 9, V79B 73 ± 17, XR-V15B 34 ± 16, XR-C1 75 ± 28, V79 62 ± 18, and V-C4 73 ± 14.

Surviving fractions \( (S[D]/S[0]) \) after dose \( D \) were calculated and survival curves were analyzed with SPSS statistical software (SPSS 9.01 for Windows, Chicago, Illinois, USA). The HDR data were fitted by a weighted linear regression according to the LQ formula:

\[
S(D)/S(0) = \exp - (\alpha D + \beta D^2)
\]

Most LDR data were fitted to a weighted pure exponential model using only the linear term: \( S(D)/S(0) = \exp - (\alpha D) \); however, for the CHO9 and the EM-C11, the LDR data were fitted to the LQ formula. XR-C1 and XR-V15B survival data were not fitted.

**RESULTS**

**Cell cycle distribution**

The doubling times of the hamster mutants used in this study during exponential growth are from 11.2 to 17.5 h. Since the maximum irradiation time (i.e., 6 hours) comprises a large part of the cell cycle time of these cells, they were always irradiated in plateau phase to minimize the effects of repopulation and cell cycle redistribution. Table 2 shows the percentage of cells in S/G0/G1 phase at the start of the irradiation.

**Cell survival**

Figure 1 shows cell survival of the parental and mutants cell lines after HDR and p-LDR irradiation. Table 3 shows the corresponding parameters of the LQ model. It was not possible to describe the survival curves of the Ku-80 and DNA-PKcs mutants with the LQ model, and therefore no data are given.

The dose-modifying factors determined at the 10% survival level after HDR irradiation for the different mutant/parent couples were 1.5 for EM-C11/CHO9, 1.3 for irst1SF/A8, 2.3 for V-C4/V79, 3.8 for XR-C1/CHO9, and 4.4 for XR-V15B/V79B.

No differences between LDR and HDR are observed for the AT-like mutant V-C4, the HR mutant irst1SF, the Ku80 mutant XR-V15B, or the DNA-PKcs mutant XR-C1, but for the parental cell lines a dose rate effect is observed. For the ssb repair mutant EM-C11 and its parental cell line, a dose rate effect is observed.

| Table 2. Cell cycle distributions of mutant cell line at the time of irradiation. |
|------------------|------------------|------------------|------------------|
|                  | G0/G1 (%)*       | S (%)*           |
| CHO9             | 82 ± 4           | 5 ± 2            |
| EM-C11           | 84 ± 2           | 8 ± 0.2          |
| XR-C1            | 84 ± 2           | 3 ± 2            |
| AA8              | 86 ± 2           | 6 ± 1            |
| irst1-SF         | 69 ± 6           | 10 ± 6           |
| V79B             | 87 ± 8           | 1 ± 0.2          |
| XR-V15B          | 72 ± 6           | 12 ± 2           |
| V79              | 76 ± 8           | 11 ± 1           |
| V-C4             | 51 ± 2           | 26 ± 3           |

*Means of at least 2 experiments ± standard error

Table 3. Linear quadratic parameters for cell survival after HDR and p-LDR irradiation.

<table>
<thead>
<tr>
<th></th>
<th>HDR (a)*</th>
<th>HDR (b)*</th>
<th>LDR (a)*</th>
<th>LDR (b)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO9</td>
<td>0.20 ± 0.04</td>
<td>0.026 ± 0.005</td>
<td>0.17 ± 0.03</td>
<td>0.0230 ± 0.004</td>
</tr>
<tr>
<td>EM-C11</td>
<td>0.09 ± 0.09</td>
<td>0.12 ± 0.02</td>
<td>0.38 ± 0.03</td>
<td>0.0320 ± 0.004</td>
</tr>
<tr>
<td>AA8</td>
<td>0.19 ± 0.03</td>
<td>0.040 ± 0.004</td>
<td>0.33 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>irst1-SF</td>
<td>0.36 ± 0.07</td>
<td>0.046 ± 0.013</td>
<td>0.71 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V79B</td>
<td>0.06 ± 0.03</td>
<td>0.027 ± 0.004</td>
<td>0.18 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>XR-V15B</td>
<td>No Fit</td>
<td>No Fit</td>
<td>No Fit</td>
<td>No Fit</td>
</tr>
<tr>
<td>CHO9</td>
<td>0.20 ± 0.04</td>
<td>0.026 ± 0.005</td>
<td>0.28 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>XR-C1</td>
<td>No Fit</td>
<td>No Fit</td>
<td>No Fit</td>
<td>No Fit</td>
</tr>
<tr>
<td>V79-2</td>
<td>0.150 ± 0.025</td>
<td>0.025 ± 0.003</td>
<td>0.17 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V-C4</td>
<td>0.59 ± 0.05</td>
<td>0.02 ± 0.01</td>
<td>0.78 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

*α (Gy⁻¹) and β (Gy⁻²)
Some of the mutant LDR survival curves lie below the HDR survival curves in the initial part (0-4 Gy dose range) of the curves (see Fig. 1). Because there is no significant difference between HDR and p-LDR irradiation for the individual survival points, this is mostly a result of the fitting procedure of the survival curves to the experimental models.

Fig. 1. Cell survival of mutant and parental cell lines after HDR and p-LDR irradiation in the plateau phase. Points represent means with standard errors of at least 3 experiments. A: SSB repair, EM-C11 and its parental cell line CHO9; B: Homologous recombination, irs1SF and its parental cell line AA8; C: NHEJ, XR-V15B, and its parental cell line V79B; D: NHEJ, XR-C1, and its parental cell line CHO9; E: AT-like, V-C4, and its parental cell line V79.
Low Dose Rate X-rays in Mutant Cells

In one case, the irs1SF mutant, the LDR curve seems to be more sensitive. Although there is a difference in time schedule, after HDR irradiation the cells were allowed 24 hours to repair before plating and after p-LDR irradiation they were plated immediately, potentially lethal damage repair is unlikely. In other experiments, there was no difference between the survival of irradiated (HDR) mutant or parent cells plated immediately or after a 24 h delay. Because of the spread of data in the survival curve of the irs1SF cells after HDR, the data could maybe as well be fitted by a straight line.

In most cell lines, exponentially growing cells are more sensitive to HDR radiation than confluent “plateau phase” cells (cf. Haveman et al., a result of the significant differences in cell cycle distribution at the time of irradiation. This is particularly so for the irs1SF cell line. The sensitivity of irs1SF cells in the plateau phase for HDR irradiation differed very significantly ($p < 0.001$) from the sensitivity of the cells in the exponential growth phase, as shown in Fig. 2. In the log phase, irs1SF is approx. 2.5 times more sensitive than in the plateau phase.

![Cell survival of the HR mutant irs1SF in the log phase and the plateau phase after HDR irradiation](Image)

**Fig. 2.** Cell survival of the HR mutant irs1SF in the log phase and the plateau phase after HDR irradiation. The points represent means with standard errors of at least 3 experiments.

**DISCUSSION**

A change in sensitivity when the dose rate was decreased was observed for all parental cell lines and the DNA ssb mutant EM-C11. For all cell lines exhibiting this dose rate effect, the effect was observed only above 4 Gy p-LDR. Below 4 Gy no differences could be observed between HDR irradiation and p-LDR irradiation.

The NHEJ mutants are the most radiosensitive cell lines used in this study. Their dose-modifying factors are much higher (DMF (DNA-PKcs mutant) = 3.8, DMF (Ku80) = 4.4) than in the other cell lines (DMF 1.5-2.75).

Both after HDR and LDR irradiation, the Ku80 mutant (XR-V15B) exhibits its characteristic biphasic survival curve first described by Zdzienicka et al. For the Ku80 and the DNA-PKcs mutants (XR-V15B and XR-C1), no dose-rate effect was observed, which suggests a defect in SLD repair. This is in agreement with several previously reported studies on the xrs cell lines (Ku80 mutants) and the irs-20 mutant (DNA-PKcs mutants).

Both Ku80 and DNA-PKcs are members of the nonhomologous end-joining pathway (Jeggo, 1998). This dsb repair mechanism plays an important role in the repair of DNA damage after ionizing radiation. The absence of a change in radiosensitivity after the reduction of the dose rate in the DNA dsb repair (NHEJ) mutants suggests that this system plays an important role in sublethal damage repair and the generation of the dose-rate effect.

The DNA ssb repair-deficient EM-C11 exhibits a dose rate effect just like its parental cell line, which indicates that these cell lines have normal SLD repair. Our results on the EM-C11 mutant are in agreement with the previously reported results on EM9, another XRCC1 mutant. This mutant was shown to exhibit normal sublethal damage repair in split-dose recovery experiments. Since it shows normal sublethal damage repair in split-dose recovery and a normal dose-rate effect as shown here, it can be concluded that ssb repair is not essential for the dose-rate effect.

The absence of a dose-rate effect in the AT-like mutant concurs with previously described results by Thacker and Wilkinson. In the irs2 mutant, another member of the XRCC8 complementation group, no cellular recovery was observed when the dose rate was reduced. This indicates that the AT-like protein is essential for sublethal damage repair.

The XRCC3 irs1SF mutant, which is impaired in homologous recombination, shows no change in sensitivity when the dose rate is reduced. In contrast, it was previously reported that the XRCC2 mutant irs1 showed considerable recovery potential when the dose rate was decreased. Both XRCC2 and XRCC3 are part of the Rad51 family and play a role in the repair of DNA dsb by homologous recombination. It is striking that in this study, XRCC3 is found to be necessary for the dose rate effect, but XRCC2 appears not to play a role. Another possible explanation for this observation could be found in the difference in the repair kinetics of both mutants; i.e., the dose rate in our study could be too high, or the time between the pulses too short to allow repair to occur in the irs1SF.

Next to the NHEJ mechanism, HR is thought to be the second repair mechanism involved in the repair of dsb. Because in our study no dose-rate effect is observed in the mutants involved in the repair of DNA dsb’s indicates that SLD repair is absent in these mutants.

The HR mutant irs1SF exhibited a clearly different radiosensitivity after HDR irradiation in exponential growth
phase compared to irradiation in the plateau phase. This is in agreement with the assumption that HR is cell cycle-dependent and is preferentially used in the late S-G2 phase when sister chromatids are close to each other\(^4\). In the plateau phase, the S, M, and G2 phase are greatly reduced, and the G1 phase is most abundant. It has been reported that in the G1/early S phase and G2, the activity of the DNA-PK complex involved in NHEJ peaks\(^4\). It is very likely that the difference in radiosensitivity between the log phase and the plateau phase in the irs1SF cells should be attributed to repair by the NHEJ pathway in the G1 phase, which is not disrupted in this mutant. From the results in this study, it is clear that cell survival after irradiation in plateau phase mutant cells is not the same as that of the parental cell line, as would be expected if HR is only functional in the S-phase of the cell cycle. Two plausible explanations exist for this observation: 1. The small population of S-phase cells still present in the plateau phase cultures “contaminate” the population, resulting in a more radiosensitive survival curve than expected. 2. HR also takes place outside the S-phase, but at a greatly diminished rate. Because mutant irs1SF cells in “plateau” are slightly more sensitive to p-LDR than to HDR irradiation might be the result of some continuous recruitment of the cells into the sensitive S-phase during the prolonged p-LDR procedure.

To summarize, we show in this study that cells mutated in DNA ssb repair have normal SLD repair after p-LDR irradiation, but the mutants involved in DNA dsb repair or exhibiting an AT-like mutation show no SLD repair, thus resulting in the absence of a dose-rate effect after p-LDR irradiation. In this study we have demonstrated that NHEJ, the XRCC3 protein of HR pathway and the AT-like protein are necessary for the dose-rate effect. Single-strand break repair probably plays only a minor role.

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