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X-Ray Induced Mutation Frequency at the *Hypoxanthine Phosphoribosyltransferase* Locus in Clinically Relevant Radioresistant Cells

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Abstract

To elucidate the molecular mechanisms underlying cellular radioresistance, clinically relevant radioresistant cell lines were established via long-term exposure to X-rays with stepwise dose escalation. Established cells continue to proliferate despite exposure to 2 Gy X-rays/day for more than 30 days, a standard protocol in cancer radiotherapy. DNA repair fidelity in radioresistant and the parental cells by evaluating the mutation frequency at the *hypoxanthine phosphoribosyltransferase* (*HPRT*) locus after exposure to X-rays was determined. Mutation spectrum at the *HPRT* locus was examined by multiplex polymerase chain reaction. Rejoining kinetics of X-ray-induced DNA double strand breaks (dsbs) was evaluated by the detection of phosphorylated histone H2AX (γH2AX) after X-irradiation. The fold increase in the *HPRT* mutation frequency due to acute radiation was similar between radioresistant and the parental cell lines. However, fractionated radiation (FR) consisting of 2 Gy X-rays/day increased the mutation frequency at the *HPRT* locus in parental but not in radioresistant cells. Analysis of the FR-induced mutations at the *HPRT* locus revealed a high frequency of deletion mutations (>70%) in parental but not in

radioresistant cells. As assessed by γH2AX immunostaining, DNA dsbs induced by acute exposure to 10 Gy of X-rays were repaired to the control level within 7 days in radioresistant but not in the parental cells. Moreover, 2 Gy × 5 FR increased the number of γH2AX-positive cells in parental cultures but not in radioresistant cultures. DNA dsbs induced by 2 Gy/day FR are repaired with fidelity in radioresistant but not in parental cells.

Keywords

Clinically Relevant Radioresistant (CRR) Cell, Hypoxanthine Phosphoribosyltransferase (HPRT), Mutation Frequency, Phosphorylated Histone H2AX (γH2AX), X-Rays

1. Introduction

In the past few decades, extensive efforts have been focused on improving cancer therapy by establishing more successful treatment approaches and enhancing early detection technology. Radiotherapy (RT) is an important approach to the treatment of localized early cancers and can be used in combination with other treatments. Recent advances in tumor imaging, physical targeting of ionizing radiation (IR) and optimization of the IR delivery have yielded significant improvements in patient outcomes. Whether inherent or induced, cellular radioresistance is a major factor in the failure of RT. The molecular basis of radioresistance remains to be elucidated. The radioresistant phenotype has been correlated with characteristics such as the enhanced repair of IR-induced DNA damage [1] [2] [3] [4], overexpression of cyclin D1 [5] [6], higher rates of cell growth and migration [7], a lower concentration of reactive oxygen species (ROS) in cells [8] [9] [10], inactivation of apoptotic proteins [11] and alterations in radiation-induced autophagy [12] [13]. A disadvantage of studying radioresistance is the lack of adequate radioresistant models reflecting the clinical features of this phenomenon. Many studies have focused on the comparison of tumors or cell lines with differing radiosensitivities to elucidate the molecular mechanisms underlying radioresistance. However, the mechanisms of radioresistance are complex and are likely to differ in cells with different origins. The inherent variation among cells with different origins makes it difficult to determine the extent to which candidate factors contribute to cellular radioresistance and to identify factors that are essential for radioresistance. Isogenic models consisting of cells from the same origin that differ only in radioresistance have been increasingly pivotal in elucidating the cellular mechanisms of radioresistance [1] [7] [10] [14]. These models avoid the influence of confounding factors, allowing one to unravel the molecular mechanisms that are truly involved in radioresistance. Conventional fractionated RT consists of 2 Gy per fraction once per day, 5 days a week for 5 - 7 weeks [15]. To elucidate the molecular mechanisms underlying cellular radioresistance, we established clinically relevant radioresistant (CRR) cell lines from several hu-

man cancer cell lines via long-term exposure to X-rays with stepwise dose escalation [1]. By definition, CRR cells continue to proliferate despite exposure to 2 Gy X-rays/day for more than 30 days. This treatment regimen is a standard protocol in cancer radiotherapy.

Studies investigating the rejoining kinetics of radiation-induced DNA double strand breaks (dsbs) suggest that radioresistant cells efficiently repair DNA dsbs [4] [16] [17]. Several reports suggest that radioresistance is associated with the enhanced repair of radiation-induced DNA damage [1] [7] [18] [19]. Our previous study also showed that X-ray-induced phosphorylated histone H2AX (γH2AX) foci disappear more rapidly in HepG2-8960-R than in parental HepG2 [1]. However, these studies focused on the rejoining kinetics of DNA dsbs but not the fidelity of DNA repair in radioresistant cells. Previous studies have demonstrated that genomic instability is usually observed in cells after irradiation [20] [21]. Morgan *et al.* proposed that increased plasticity of the genome contributes to cellular radioresistance [22]. Taken together, these studies suggest that radiation-induced DNA dsbs are rapidly rejoined with some mistakes in radioresistant cells.

Hypoxanthine guanine phosphoribosyl transferase (HPRT) plays a role in the purine salvage pathway. In addition to its normal substrates, HPRT catalyzes the phosphoribosylation of the purine analogue 6-thioguanine (6-TG), rendering this molecule cytotoxic to normal cells. In contrast, cells with mutations in the HPRT gene cannot catalyze 6-TG and survive treatment with this molecule. HPRT is a single copy gene located on the X-chromosome, and thus one copy is present in male cells. The mono-allelic nature of HPRT expression is convenient for measuring mutation frequencies and types [23]. We hypothesized that X-ray-induced DNA dsbs were repaired with greater accuracy in CRR cells compared with parental cells. In this study, we attempted to validate this hypothesis by evaluating the mutation frequency at the *HPRT* locus after exposure to X-rays.

2. Materials and Methods

2.1. Cell Culture

HepG2, SAS and HeLa human cancer cell lines were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. The HepG2-8960-R, HepG2-R, SAS-R and HeLa-R CRR cell lines were independently established from the parental HepG2, SAS and HeLa cell lines through a stepwise increase in X-ray dose exposure from 0.5 to 2 Gy/day [24]. The cells that continued to stably proliferate under exposure to 2 Gy/day of X-rays were defined as CRR cells. To maintain the CRR phenotype, the cells were exposed to FR consisting of 2 Gy X-ray every 24 hours. All of the cells used in this study were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 5% fetal bovine serum (Gibco Invitrogen Corp., Carlsbad, CA, USA) in a humidified atmosphere containing 5% CO_2 at 37˚C. Acute radiation (AR) exposure experiments were performed using cells in the exponentially growing phase. CRR cells were

exposed to AR 24 hours after the last maintenance irradiation.

2.2. Irradiation

X-irradiation was performed using a 150-KVp X-ray generator (Model MBR-1520R, Hitachi, Tokyo, Japan) with a total filtration of 0.5 mm aluminum plus 0.1 mm copper at a dose rate of 1.0 Gy/min. In this study, 2 different modes of irradiation were performed: FR and AR. The conditions for FR were treatment with 2 Gy of X-rays every 24 hours unless otherwise noted.

2.3. Selection of HPRT-Mutant Clones and Determination of Cell Survival

Mutation frequencies were calculated according to Kubota *et al.* with some modifications [25]. Prior to commencing the X-ray exposure experiments, spontaneously induced HPRT-mutant cells were eliminated by culturing cells for 1 month in hypoxanthine aminopterin thymidine (HAT) medium (Gibco Invitrogen Corp.). Following X-irradiation, the cells were incubated for 3 days in normal medium to allow phenotypic expression. The cells (5×10^5 cells/dish) were treated with 6-TG (5 μg/ml, Tokyo Chemical Industry Ltd., Tokyo, Japan) in a 10-cm dish (TPP, Tanner Plastic Products AG, Trasadingen, Switzerland) until colonies became visible. Each HPRT-mutant clone was carefully picked up under a microscopy and was individually cultured in a new 35-mm dish to obtain adequate amount of DNA for mutation analysis. Spontaneously induced HPRT-mutants were collected using the same procedure. The data are expressed as the mutation frequency ±95% confidence interval calculated by Microsoft Excel 2010 (Microsoft, Tokyo, Japan). Cell survival was measured by incubating 2×10^3 cells/60-mm dish for 1 - 2 weeks.

2.4. Multiplex PCR Analysis of Genomic DNA Isolated from the HPRT-Mutants

A DNeasy Blood & Tissue Kit (Qiagen, Chatsworth, CA, USA) was used to extract genomic DNA from the cells. All of the exonic regions of the *HPRT* locus excluding exon 1 were amplified by multiplex PCR. The PCR conditions and primer sequences were essentially as the same as the methods described by Kagawa *et al.* [26]. Ex Taq DNA polymerase was used to amplify the genomic DNA (Takara Bio Inc., Shiga, Japan). The PCR primers were purchased from Nihon Gene Research Laboratories Inc. (Sendai, Japan). All of the PCR products were analyzed by electrophoresis in a 2% agarose gel (Nacalai Tesque Inc.). For internal control, a fragment of the β-actin gene was amplified. The primer set was forward: CGT CTT CCC CTC CAT CG and reverse: CTC GTT AAT GTC ACG CAC.

2.5. Quantitative Analysis of γH2AX-Positive Cells after X-Irradiation

To quantitate the cells with DNA dsbs, the frequency of γH2AX-positive cells present

after irradiation was determined using a MUSE H2A.X Activation Dual Detection Kit according to the manufacturer's protocol (Merck Japan Ltd., Tokyo, Japan). The kit includes two directly conjugated antibodies, a phospho-specific anti-γH2AX (Ser139)-Alexa Fluor 555 antibody and an anti-Histone H2AX-PE-Cy5-conjugated antibody designed to measure the extent of phosphorylated γH2AX relative to the total H2AX expression [27].

2.6. Statistical Analysis

The results are expressed as the mean ± standard deviation (S.D.) of three independent experiments. Statistical significance was determined with a two-tailed Student's *t*-test.

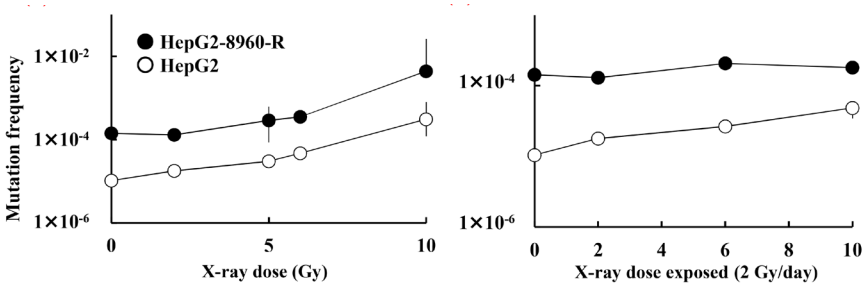
3. Results

3.1. Mutation Frequency at the *HPRT* Locus in HepG2-8960-R and HepG2

The basal mutation frequency at the *HPRT* locus was 10 times higher in HepG2-8960-R than in HepG2 (**Figure 1(a)**). Exposure to AR increased the mutation frequency at the *HPRT* locus in a dose-dependent manner in both HepG2-8960-R and HepG2. On the other hand, the mutation frequency induced by FR was different between HepG2-8960-R and HepG2. The mutation frequency of HepG2 increased to 5 fold after exposure to 5 FR but that of HepG2-8960-R was fairly constant even after exposure to a total of 10 Gy of X-rays (**Figure 1(b)**).

3.2. Mutation Spectrum at the *HPRT* Locus

At least 10 mutants were evaluated for the presence or absence of exons after exposure to AR or FR. A representative profile obtained from the multiplex PCR is shown in **Figure 2(a)**. Clone No. 6 exhibits a total deletion from exon 2 to exon 9, and clone No. 12 exhibits a partial deletion from exon 5 to exon 9. In contrast, clone No. 2 has point mutations or a small deletion that is unidentifiable with the PCR



(a) (b)

Figure 1. The mutation frequency at the *HPRT* locus in HepG2-8960-R (●) and HepG2 (○) after exposure to a single dose or fractionated dose (2 Gy/day) of X-rays. (a) The mutation frequency at the *HPRT* locus after exposure to a single dose of X-rays. The results are expressed as the mutation frequency ±95% confidence interval; (b) The relative mutation level at the *HPRT* locus after exposure to a single dose of X-rays.

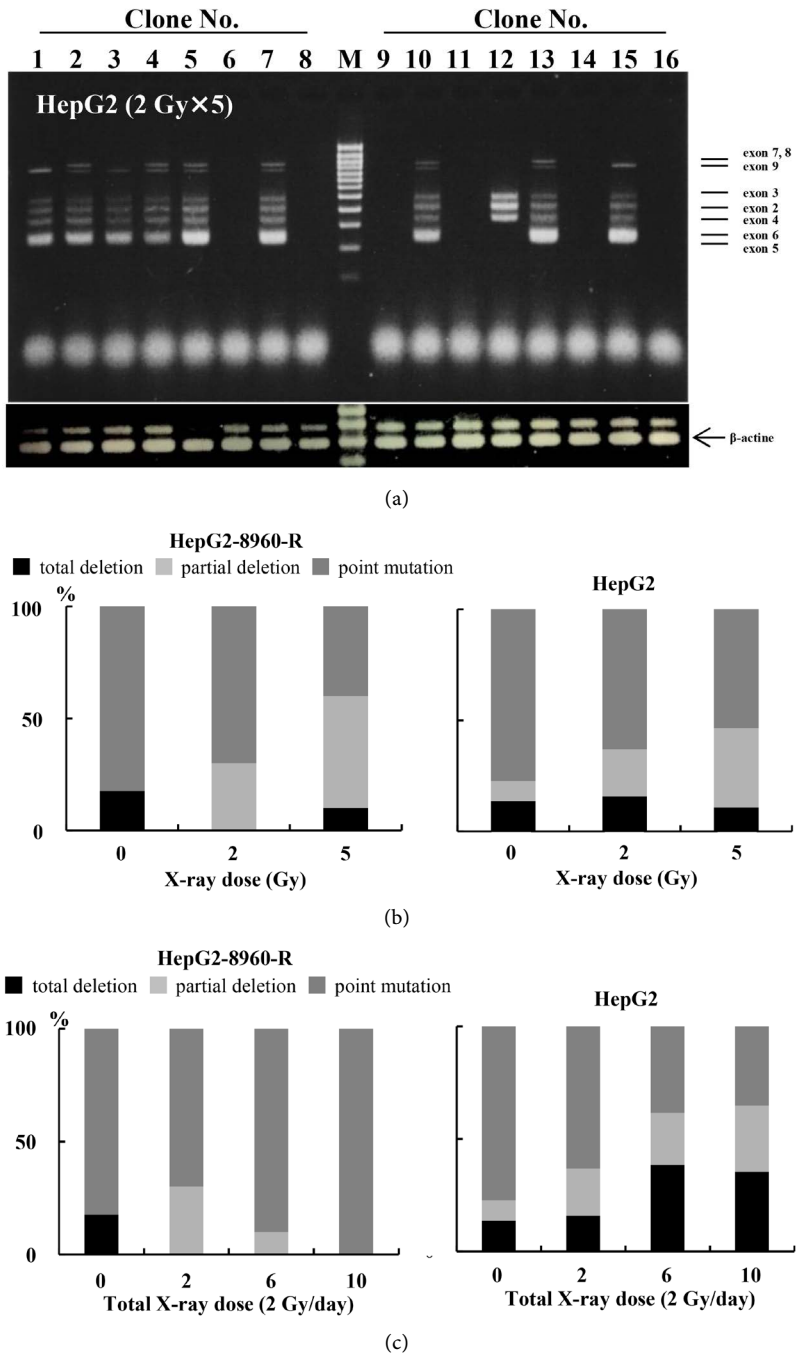


Figure 2. Mutation patterns at the *HPRT* locus after exposure to a single dose or fractionated dose (2 Gy/day) of X-rays. (a) A representative image of an agarose gel showing the PCR products of the HepG2 *HPRT* mutant clones exposed to 2 Gy × 5 (total 10 Gy) of X-rays. The *HPRT* exon number is indicated on the right. M: DNA size marker; (b) The *HPRT* mutation patterns in HepG2-8960-R and HepG2 after treatment with 0, 2 and 5 Gy of X-rays; (c) The *HPRT* mutation patterns in HepG2-8960-R and HepG2 after treatment with fractionated X-rays (2 Gy/day).

methods used in this study. The quality and quantity of genomic DNA obtained from the samples were tested by the presence of β-actin fragments.

The HPRT mutants were classified as total deletions, partial deletions and point mutations that might contain a small deletion. The results are summarized in Figure 2(b), Figure 2(c) and Table 1. Point mutations were the most frequent (>70%) spontaneously occurring mutations in both HepG2-8960-R and HepG2. AR exposure dose-dependently increased the frequency of partial deletions in both HepG2-8960-R and HepG2 (Figure 2(b)). Exposure to 5 Gy of AR did not increase the frequency of total deletions in HepG2-8960-R or HepG2. FR exposure increased the frequency of both partial and total deletions in HepG2 but not in HepG2-8960-R (Figure 2(c)). The spontaneous deletion rates in HepG2-8960-R and HepG2 were 17.6% and 22.7%, respectively. No deletions were induced by exposure to 5 × 2-Gy FR in HepG2-8960-R; however, the deletion rate was 64.7% in HepG2 after exposure to the same dose of FR.

3.3. Rejoining Kinetics of X-Ray-Induced DNA DSBs

The cellular level of γH2AX (as detected by MUSE) correlated with the number of DNA strand breaks, the amount of cell death and radiosensitivity. H2AX is phosphorylated in response to dsbs. Knowledge of the causes of dsbs will improve our understanding of the mechanisms involved in the DNA damage response and subsequent repair of these breaks. More than 90% of CRR and parental cells were γH2AX-positive 1 hour after exposure to 10 Gy of AR, and the frequency of γH2AX-positive cells did not differ significantly between the two cell lines (Figure 3). The fraction of γH2AX-positive cells decreased more rapidly in the CRR cells compared with the parental cells. Seven days after exposure to 10 Gy of AR, the frequency of γH2AX-positive radioresistant cells returned to the control level in CRR cells but remained at significantly higher levels in the parental cells.

To investigate whether FR affects CRR cells and parental cells in the same manner, we compared γH2AX accumulation by sequential treatment with FR. FR treatment increased the frequency of γH2AX-positive parental cells but not CRR cells (Figure 4). After exposure to 7 × FR (14 Gy total), approximately 70% of the parental cells were γH2AX-positive. In contrast, exposure to the same dose of FR (7 × FR of X-rays) did not increase the frequency of γH2AX-positive CRR cells. We speculated that homologous recombination (HR) is more active in CRR cells than in parental cells. However, immunocytochemistry of RAD51 after irradiation revealed that the average number of foci per nucleus between CRR and parental cells was not significantly different (data not shown).

4. Discussion

Previous studies have suggested that radioresistant cells rejoin DNA dsbs more efficiently than radiosensitive cells [4] [17] [28]. However, it is unclear whether radiation-induced DNA dsbs in radioresistant cells are repaired accurately. The potential role of initial DNA damage or the rejoining kinetics of DNA dsbs after irradiation in cellular radiosensitivity is also controversial [4] [29] [30]. Olive *et al.* showed that the rejoining rate or amount of residual DNA damage after exposure

Table 1. Classification of exon deletions in the HPRT locus.

	HepG2 (Clone No.)	Exon								Number of clones examined
		2	3	4	5	6	7	8	9	
Control	7	■	■							21
	9	■	■	■	■	■				
2 Gy	4						■	■	■	23
	12			■						
	13						■	■		
	18						■	■		
5 Gy	1	■	■	■						28
	8						■	■		
	9						■	■		
	10						■	■		
	11						■	■		
	12						■	■		
	14						■	■		
	16						■	■		
	17						■	■		
	20						■	■		
2 Gy × 3	9						■	■		13
	10						■	■		
	12						■	■		
2 Gy × 5	1						■	■		18
	3						■	■		
	12				■	■	■	■	■	
	15						■	■		
	17						■	■		
	HepG2-8960-R (Clone No.)									
Control										17
2 Gy	2						■	■	■	20
	3						■	■	■	
	4						■	■	■	
	6						■	■	■	
	7						■	■	■	
	15								■	
5 Gy	3				■					12
	8						■	■		
	9			■	■	■	■	■	■	
	11				■					
	12				■					
2 Gy × 3	5				■					20
	7				■					
2 Gy × 5										20

Black boxes indicate exon regions of HPRT that were not amplified by multiplex PCR. In this table clones with no observable deletion by PCR and that with total deletion were not presented. The number of clones with total deletion was as followed; HepG2, 0 Gy, 3 clones, 2 Gy, 3 clones, 5 Gy, 3 clones, 2 Gy × 3, 5 clones, 2 Gy × 5, 6 clones. HepG2-8960-R, 0 Gy, 3 clones, 2 Gy, 0 clone, 5 Gy, 1 clone, 2 Gy × 3, 0 clone, 2 Gy × 5, 0 clone.

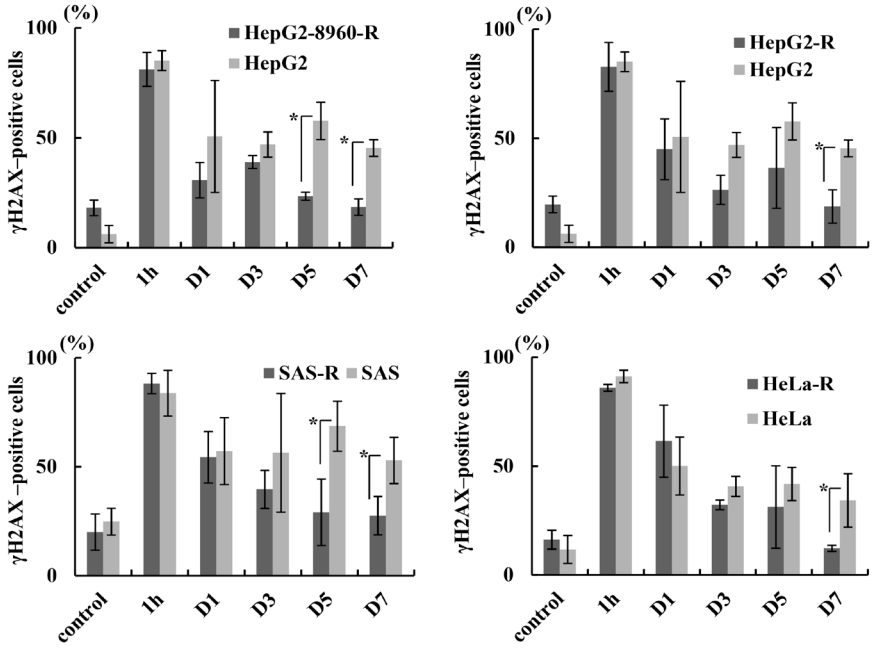


Figure 3. The rejoining kinetics of X-ray-induced DNA dsbs as determined by γH2AX levels. The temporal pattern of the frequency of γH2AX-positive cells after acute exposure to 10 Gy of X-rays. Mean ± S. D. of 3 independent experiments. *; $p < 0.05$.

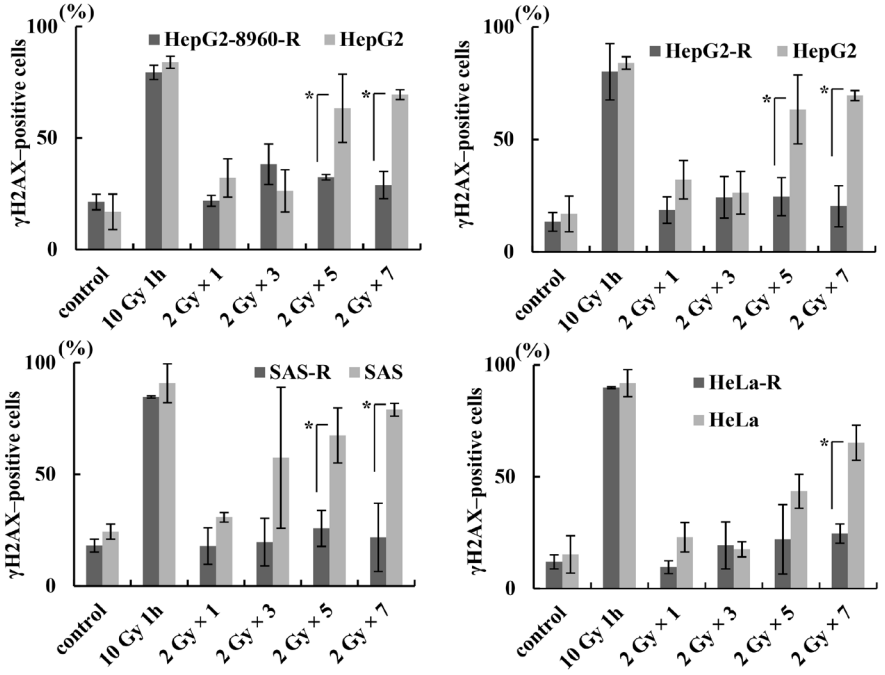


Figure 4. The rejoining kinetics of X-ray-induced DNA dsbs, as determined by the detection of γH2AX foci. The γH2AX induction frequencies in clinically relevant radioresistant cells and their corresponding parental cells 24 hours after exposure to fractionated X-rays (2 Gy/day). Mean ± S. D. of 3 independent experiments. *; $p < 0.05$

to radiation did not correlate with the cellular radiation sensitivity [31]. However, Lynam-Lennon *et al.* demonstrated that radioresistant esophageal adenocarcino-

ma cells displayed greater repair of radiation-induced DNA damage than control cells [4]. The discrepancies in these observations arise from the fact that many studies have used cell lines with differing degrees of radiosensitivity and have compared cells with different genetic backgrounds [32]. To elucidate whether DNA repair fidelity is involved in cellular radioresistance, we estimated the mutation frequency and analyzed the mutation spectrum at the *HPRT* locus in HepG2-8960-R and HepG2 after exposure to AR or FR. It would be convincing if common mutation characteristics were found in a number of CRR cell lines in addition to HepG2-8960-R.

The baseline mutation frequency at the *HPRT* locus was higher in HepG2-8960-R than in HepG2 (**Figure 1(a)** and **Figure 1(b)**). We assumed that this phenomenon was attributable to the maintenance FR provided to the HepG2-8960-R. To eradicate *HPRT* mutants prior to assessing the mutation frequency, HepG2-8960-R were cultured for 3 weeks in medium containing HAT under maintenance FR. Subsequently, the cells were cultured for an additional 1 week without receiving maintenance FR in medium containing HAT. However, we failed to completely eliminate cells carrying *HPRT* mutations. We believe that this phenomenon can be attributed to the occurrence of delayed mutagenesis due to the genomic instability induced in radioresistant cells [22] [33] [34]. We detected a higher level of ROS in the cytoplasm of HepG2-8960-R compared with HepG2 (data not shown). ROS are thought to be involved in genomic instability [35] [36] [37]. In the present study, approximately 80% of the mutations that occurred in HepG2-8960-R without irradiation were point mutations (**Figure 2**). Point mutations are difficult to identify with comparative genomic hybridization and are the predominant type of mutation resulting from genomic instability [20]. These findings strongly suggest that the undetected genomic instability in HepG2-8960-R resulted in a high baseline mutation frequency. The fold increase in mutation frequency after exposure to AR and normalization to the baseline mutation frequency was nearly the same in HepG2-8960-R and HepG2. In contrast, FR treatment increased the mutation frequency in HepG2 but not in HepG2-8960-R. Radiation-induced *HPRT* mutants are generally assumed to arise directly from DNA damage; that is, these mutations are misrepaired within a few hours after X-irradiation [38]. These results suggest that CRR cells are able to accurately repair FR-induced DNA dsbs within a few hours of each irradiation.

In the present study, the spectrum of X-ray-induced mutations obtained at the *HPRT* locus indicated that the repair response to AR-induced DNA dsbs is similar in HepG2-8960-R and HepG2 (**Figure 2(b)**). However, the cellular response to FR was different; specifically, FR increased the frequency of deletion mutations in HepG2 but not in HepG2-8960-R (**Figure 2(c)**). These observations strongly suggest that FR-induced DNA dsbs are repaired accurately in CRR cells.

The number of initial DNA dsbs present after irradiation has been suggested to be higher in radiosensitive compared with radioresistant cells [16] [30]. The neutral comet assay revealed that the initial number of DNA dsbs induced by AR treatment consisting of 30 Gy of X-rays was almost identical between CRR and

parental cells (data not shown). This observation was also supported by the frequency of γH2AX-positive cells present after exposure to 10 Gy of AR (**Figure 3**). While the tail moment returned to the control level both in CRR and parental cells 6 hours after exposure to X-rays, the rejoining kinetics for the X-ray-induced DNA dsbs were completely different in CRR and parental cells; specifically, the rejoining kinetics were monophasic in CRR cells but biphasic in parental cells (data not shown). The two major pathways for repairing DNA dsbs are HR and nonhomologous end joining (NHEJ). Of these two pathways, HR leads to accurate repair [39]. We speculate that HR is more active in CRR cells than in parental cells. RAD51 is involved in the HR pathway [40]. However, immunocytochemistry revealed that the number of RAD51 foci present in CRR and parental cells after irradiation did not differ significantly (data not shown). Further studies are required to elucidate which repair pathway is activated in CRR cells.

We further examined the repair kinetics of X-ray-induced DNA dsbs by determining the level of γH2AX (**Figure 3**). One hour after exposure to 10 Gy of AR, approximately 90% of both CRR and parental cells were γH2AX-positive. Our data are inconsistent with a previous report, in which the number of DNA dsbs induced by radiation was lower in radioresistant cells compared with radiosensitive cells [30]. This discrepancy might be explained by the use in different studies of cell lines with different genetic backgrounds to compare DNA dsbs after irradiation. For example, El-Awady *et al.* used four mammary, one bladder, two prostate, one cervical, and one squamous cell carcinoma to determine whether cellular radiosensitivity is related to the number of initial dsbs induced by X-rays [30]. Our isogenic models revealed that the initial number of X-ray induced DNA dsbs is equivalent in radioresistant CRR cells and parental cells.

Seven days after AR, approximately 50% of the parental cells remained γH2AX-positive, whereas the frequency of γH2AX-positive CRR cells had returned to the basal level (**Figure 3**). These results strongly suggest that X-ray-induced DNA dsbs are repaired more efficiently in CRR cells compared with parental cells. In contrast to the parental cells, the frequency of γH2AX-positive CRR cells did not increase, even after 7 days of FR. These results suggest that CRR cells are able to repair the DNA dsbs induced by a moderate dose of X-rays (such as 2 Gy in 24 hours).

5. Conclusion

CRR cells repair DNA dsbs more accurately than parental cells. The repair kinetics and decreased frequency of γH2AX-positive cells after exposure to FR of 2 Gy/day is a powerful predictor of the CRR phenotype. This is the first study to show the DNA repair fidelity of radioresistant cells using an isogenic model. The possible activation of the HR pathway to repair dsbs in CRR cells was eliminated by immunostaining for RAD51 foci. Our previous data showed that the number of cells in the G2 phase of the cell cycle is more abundant in CRR cells than in parental cells [1]. High-fidelity NHEJ in G2 might be involved in DNA repair in CRR cells. To confirm this hypothesis, additional analyses using the I-SceI sys-

tem [41] are currently underway in our laboratory.

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