Split Dose Recovery Studies using Homologous Recombination Deficient Gene Knockout Chicken B Lymphocyte Cells

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INTRODUCTION

All normal mammalian cells exhibit innate capacities to recover from both endogenous and exogenous insults, including ionizing radiation. Irradiation of mammalian cells produces a variety of lesions in DNA, such as the base damage, strand breaks and cross links within the DNA or between DNA and nuclear proteins. Among these, the double strand break (DSB) is considered to be the critical lesion for radiation induced cell death.1–5 Deficiency in DSB repair or inhibition of DSB repair results in higher rates of mammalian cell death, as evidenced by a number of studies on radiosensitive DSB repair-deficient mutant cells1,6,7 and cells from scid mice.8

The ability of mammalian cells to repair sub-lethal damage (SLD) induced by ionizing radiation is very well established both experimentally and clinically.9–14 Several reports have documented the inability of repair of SLD in cells.15–17 These cells are defective in the catalytic subunit of DNA dependent protein kinase (DNA-PKcs) or the DNA-binding sub unit of DNA-PK, which are involved in repair of DNA strand breaks. SLD repair has an important role in radiotherapy through fractionation, as radiation is generally given in multiple fractions of low doses to allow the normal cells to recover from radiation damage, while attaining maximum damage to tumour cells.

It is clear from the earlier studies that, basically two pathways namely, homologous recombination (HR) and non-homologous end-joining (NHEJ) are operating in eukaryotic...
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Several regulatory proteins involved in the DSB repair mechanism have also been identified. It is also known that the X-ray repair cross complementing (XRCC) genes correct the phenotypes of certain mutant rodent cell lines that are sensitive to DNA damaging agents including ionizing radiation. Recently, the sequence analysis of cloned human XRCC2 and XRCC3,21–23 have revealed its homology to the Rad51 of yeast and mammals.22,23 Although, a few studies have demonstrated the involvement of RAD52, XRCC2, XRCC3, RAD51C and RAD51D in DSB repair through homologous recombination in mammalian cells, the knowledge accumulated is far from clear understanding. The molecular mechanism of SLD recovery is also not completely understood. However, our recent studies on SLD recovery, using chicken B lymphocyte cell line DT40 (wild type) cells and its RAD54, RAD54/KU70, KU70 gene knockout cells after treatment with X-rays, showed that the repair of sublethal damage is due to DSB repair mediated by homologous recombination.24 The present study using chicken DT40 cells proficient or deficient in the above key proteins further broadens our understanding on the role of RAD52, XRCC2, XRCC3, RAD51C and RAD51D in DSB repair mechanism in influencing the SLD recovery.

MATERIALS AND METHODS

Cells and cell culture conditions

Generation of RAD52−/−, XRCC2−/−, XRCC3−/−, RAD51C−/−, RAD51D−/−, RAD54−/− DT 40 cells have been explained elsewhere.25–27 The above cell lines as well as the control DT 40 cells were maintained as suspension cultures in alphamEM medium supplemented with mercaptoethanol (10 µM), penicillin (100 µM/ml), streptomycin (100 µM/ml), 10% fetal bovine serum (FBS, Hyclone, London, UK) and 1% chicken serum (Sigma, USA) in a humidified atmosphere of 5% CO2 in air at 39.5°C.

Irradiation and cell survival assay

Exponentially growing cells in suspension were diluted to get appropriate cell counts (2 × 105 cells/ml), divided into various groups and exposed to X-rays. Radiation was administered using a X-ray machine (Softex Co., Tokyo, Japan) at a dose rate of 7.5 cGy/sec, with a 2.0 mm Al filter, operating at 150 kVp and 20 mA. For the split dose recovery studies, a first dose which kills approximately 90% cells was selected from the dose response curve of various cells used in this study. X-irradiation was given as two equal fractions separated by repair intervals of 0–24 h at 39.5°C. Immediately after the treatment, serially diluted cells were plated in triplicate petri dishes with 6 ml of 1.5% (w/v) methylcellulose (Aldrich, Milwaukee, WI) containing D-MEM/F-12 (Gibco-BRL), 5% chicken serum and 10mM beta-mercaptoethanol. Colonies were counted 8–10 days after plating. Percentage survival was calculated relative to the number of colonies from the control.27 All experiments were repeated 2–3 times to check for consistency.

Fig. 1. Survival curves of exponentially growing chicken B-cell lines, DT40, RAD54−/−, RAD52−/−, XRCC2−/−, XRCC3−/−, RAD51C−/− and RAD51D−/−. Two dotted lines are DT40 and RAD54−/− cells that already published (with permission, Utsumi et al. 2001). Cells were irradiated in suspension at a concentration of about 2 × 105 cells/ml of growth medium at room temperature. The cells growing as suspension cultures were then transferred to CO2 incubator at 39.5°C for the time interval indicated, diluted and plated in methylcellulose medium for colony formation. Plating efficiencies of DT40, RAD54−/−, RAD52−/−, XRCC2−/−, XRCC3−/−, RAD51C−/− and RAD51D−/− cells are about, 98, 70, 95, 82, 76, 90 and 89% respectively. Each survival curve consists of data from three to four independent experiments and each point were Mean ± SE.
Fig. 2. Surviving fractions of exponentially growing chicken B-cell lines, DT40, RAD52\(^{-/-}\), XRCC2\(^{-/-}\), XRCC3\(^{-/-}\), RAD51C\(^{-/-}\) and RAD51D\(^{-/-}\) cells after single-dose and two-dose X-irradiation. The first dose of the two-dose exposure was given together with the exposure for the single-dose experiment for the same cell suspensions. After an appropriate time interval, a part of the cell suspension was used for the single-dose experiment and the rest of the samples were exposed to second dose of X-irradiation (doses same as the first). The first and the second doses of X-irradiation are indicated inside each panel of the graphs. Other details as in Fig. 1.
**Flow cytometry**

Immediately after the second dose exposure to X-ray, cells were either used for clonogenic assay or fixed in cold 70% ethanol and kept overnight refrigerated at 4°C. Subsequently, cells were processed for univariate DNA analysis using Cycle Test™ PLUS DNA reagent Kit (Becton Dickenson, USA). Briefly, cells (5 x 10^5) were treated with RNase A and stained with propidium iodide (PI). PI was excited with 488 nm laser line from a 15 mW air-cooled argon ion laser and PI fluorescence was collected with a 640 long-pass optical filter. Flow cytometric acquisition of cells was performed using Cell Quest software on a FACScan flow cytometer and data were analysed with ModFit software (Becton Dikenson, Mountain View, CA).

**RESULTS**

The radiation dose response survival curves for exponentially growing parental DT40 and its genetically knocked out cell lines viz., RAD54^{+/–}, RAD52^{+/–}, XRCC2^{+/–}, XRCC3^{+/–}, RAD51C^{+/–} and RAD51D^{+/–} are given Fig. 1. The two dotted lines are data of DT40 and RAD52^{+/–} cells published earlier. All the knockout cells showed increased radiation sensitivity compared to the parental DT40 cells. Among the cell lines studied, RAD54^{+/–} was found to be most radiosensitive, followed by RAD51C^{+/–}, XRCC2^{+/–}, XRCC3^{+/–}, RAD51D^{+/–} and RAD52^{+/–}. Survival curves of both DT40 and RAD52^{+/–} cells had a big shoulder while the curves of other cells exhibited small shoulders. At the higher doses of radiation, RAD51C^{+/–} cells displayed higher radiosensitivity comparable to the data obtained for the HR deficient RAD54^{+/–} cells. The cell survival of RAD51D^{+/–} at the lower doses of radiation was comparatively higher and at higher doses of radiation, cell survival was lower than that of DT40 cells.

Figure 2 shows the SLD recovery kinetics in asynchronous log phase cultures of RAD52^{+/–}, XRCC2^{+/–}, XRCC3^{+/–}, RAD51C^{+/–} and RAD51D^{+/–} cells. Since these cells have differing radiosensitivity, each cell line received a different total radiation dose to yield approximately similar initial surviving fractions (approximately 10^-1). The data of Fig. 2 were obtained from three independent experiments for each cell line. All the cells used in this study demonstrated a typical split-dose recovery capacity with a peak as a function of the time after the first dose. After the split dose treatment, the survival of DT40 and RAD52^{+/–} cells increased until a maximum was reached at about 1.5 h and decreased to a minimum thereafter (4–4.5h), with a subsequent gradual increase in cell survival indicated as a second peak at 8 h. Similarly, the knockout cells, XRCC2^{+/–}, RAD51C^{+/–} and RAD51D^{+/–} exhibited increased cell survival after split dose treatment as that of DT40 and RAD52^{+/–} cells, however there was a difference in the initial peak in cell survival which occurred at about 2–4 h, with a subsequent decrease. While for XRCC3^{+/–} cells peak in the cell survival was not obvious. The secondary moderate increase in the cell survival in these cell lines was much slower and delayed until 10–12 h when compared with that of DT40 and RAD52^{+/–} cells (8h), probably an indication of delayed progression through the cell cycle (Fig. 5).

**Fig. 3.** Survival ratios of exponentially growing DT40, RAD52^{+/–}, XRCC2^{+/–}, XRCC3^{+/–}, RAD51C^{+/–} and RAD51D^{+/–} cells. Survival ratios were calculated as the ratios of the surviving fractions from the split-dose experiments divided by the surviving fractions from the single dose experiments (from the Fig. 2). The survival ratios were normalized to unity at time 0 h for each group. Other details are as in Fig. 1.
Fig. 4. Survival curves of exponentially growing DT40, RAD54−/−, RAD52−/−, XRCC2−/−, XRCC3−/−, RAD51C−/− and RAD51D−/− cells after fractionated irradiation. Fixed first dose for DT40, RAD54−/−, RAD52−/−, XRCC2−/−, XRCC3−/−, RAD51C−/− and RAD51D−/− was 433, 215, 474, 295, 454, 266 and 370 cGy respectively. Cell incubated at 39.5°C in a humidified atmosphere of 5% CO₂ with 1.5 h interval between fixed first dose and graded second dose. DT40 and RAD54−/− data were reproduced with permission (Utsumi et al., 2001, Radiation Research).
Fig. 5. Cell cycle analysis of DT40, RAD52−/−, XRCC2−/−, XRCC3−/−, RAD51C−/− and RAD51D−/− cells. Samples used for the cell cycle studies are from the split dose experiments described in Fig. 2 and data points are from a single representative experiment.
Figure 3 shows the survival ratios of split dose versus single dose cell survival derived from Fig. 2. Lower survival ratios were observed for the most radiosensitive RAD51C−/−, XRCC2−/−, XRCC3−/− and the survival ratios were higher for the parental DT40, RAD52−/− and RAD51D−/− at the time when peak survival was noticed indicating least radiosensitivity (Figs. 1 and 3).

The results of the experiment to understand the ability of the cells to repair SLD and to check whether the reappearance of the shoulder during the split-dose interval was due to SLD repair were summarized in Fig. 4. In this experiment, to obtain the single dose cell survival curves, cells were exposed to graded doses of X-rays. To check the reappearance of the shoulder indicating the increased cell survival the respective single doses were given as two split doses with the incubation of cells at 39.5°C for 1.5 h between the two doses. There was remarkable difference with respect to the extent of reappearance of the shoulder between different cell lines. The reappearance of the shoulder was reduced in RAD51D−/− and XRCC2−/− cells when compared to the other cells (Fig. 4). There was complete absence of reappearance of shoulder for HR deficient RAD54−/− cells exhibiting hyper-radiosensitivity as shown in our earlier studies.

The data illustrated in Fig. 5 summarizes the cell cycle distribution of asynchronously growing log phase cells when exposed to first and second X-ray doses (as indicated in Fig. 2) with an interval of 0–24 h. It is implied that the perturbations observed in the cell cycle is basically due to the first dose by itself, as the cells were fixed immediately after the second dose for cell cycle analysis by flow cytometry. Immediately after the first dose of irradiation, cells progress slowly through the cell cycle as indicated by the significant initial increase in the S-phase cells, finally displayed as G2/M block by about 5 h of post-treatment which persists for some time. The subsequent release of the cells was noticed thereafter as a slow increase in the G0-G1 population of cells and a slow decrease in the G2/M population. In general, no cell line exhibited G1 block upon irradiation. The kinetics of cell cycle progression after X-irradiation was almost similar in all the cells studied, with slight differences in the passage through various phases of the cell cycle. In the mutant RAD52−/−, RAD51D−/− and parental DT40 cells, the development of the G2/M block was comparatively slower, reaching the peak at about 8–9 hours of post irradiation, but persisted for a longer time. However, XRCC2−/−, XRCC3−/− and RAD51D−/− cells exhibited an early G2/M block peaking at about 4–5 hours itself, followed by a sharp decrease in the percentage of G2/M cells indicating release of the radiation induced block.

**DISCUSSION**

The complexity of factors and mechanisms responsible for the phenotypic expression of radiation sensitivity or resistance makes it difficult to convincingly identify a particular pathway or factor. The present study was conducted using defined genetic system such as cells deficient in specific factors (like RAD52, XRCC2, XRCC3, RAD51C and RAD51D) which are involved in homologous recombination in mammalian cells. These factors are important in DSB repair through homologous recombination and thus influence the cellular response to radiation induced damage. In the present study, the cellular repair was assessed by split-dose recovery assay using the parental DT40 chicken B lymphocyte cell line and its genetically knockout cells.

It is evident from the present data that not all these knockout cells are equally proficient in SLD recovery as indicated by the difference in the initial rate and extent of recovery. The enhancement ratios obtained in the present study are well within the range obtained in earlier studies described by Hall et al.,14) for human tumor cell lines and other studies.15) For DT40, RAD52−/− and RAD51D−/− cells, a maximum enhancement ratio of 4.5–8 was observed for a split dose interval of 1.5–8 h. The relative small recovery observed in XRCC2−/−, XRCC3−/− and RAD51C−/− cell lines can be considered indicative of a lack of significant capacity for SLD repair. Alternatively, the smaller equitoxic doses used for XRCC2−/−, XRCC3−/− and RAD51C−/− cell lines might have generated a smaller number of sublethal events compared to other cells. Peacock et al.,28) demonstrated radiation dose dependent increase in the corresponding recovery ratio in human tumor cells. In our study, the significantly reduced repair response could be due to the smaller radiation dose used to irradiate RAD51C−/− cells (238 cGy). Our earlier findings with RAD54−/− chicken B lymphocyte cells have clearly demonstrated the influence of RAD54 proteins in the DSB repair through HR pathway with complete lack of split dose recovery in this cell type.24) Moreover, RAD54−/− cells show hypersensitivity to radiation exposure exhibiting a maximum radiosensitivity among all these cell lines used in this study. The knockout cells, RAD52−/−, XRCC2−/−, XRCC3−/−, RAD51C−/− and RAD51D−/− display intermediate radiosensitivities, falling in between the most sensitive RAD54−/− cell line and the least sensitive parental DT40 cells. It has been inferred by the complete absence of SLD recovery in the RAD54−/− disrupted cells that DT40 cells use preferentially HR pathway for DSB repair.24) It can be speculated from our findings that, although earlier studies substantiated the involvement of RAD52, XRCC2, XRCC3, RAD51C and RAD51D in the DSB repair by HR pathway, the role of these proteins are not as critical as that of RAD54 in DT40 chicken B cell lines.

The changes in the cell survival seen in these cells during the interval between split dose can be attributed to the redistribution of the cells in the different phases of the cell cycle after the first dose,41) and this is substantiated by the cell cycle studies in the present study. Factors such as repair rate29) and its fidelity30,31) may also influence the radiosensi-
tivity.

The XRCC2 and XRCC3-mutant cell lines irs1 and irs1SF showed only moderate hypersensitivity to ionizing radiation and no measurable defect in post-irradiation DSB repair. The present study supports this observation as there is a substantial recovery observed in the split dose recovery studies of corresponding XRCC3−/− and XRCC2−/− knockout cells.

In yeast (S.cerevisiae), members of the Rad51 gene family have been shown to play critical roles in repair of DNA double-strand breaks (DSB) through homologous recombination. HR pathway is evidently most efficient during S and G2 phases of the cell cycle, when a sister chromatid is available as a template. It is also observed that in both diploid and haploid state, yeast cells are more resistant to ionizing radiation during these phases of the cell cycle. Apparently normal radiosensitivity of RAD52−/− cells observed in the present study corroborate the earlier findings. In conditional gene knockout DT40 chicken lymphoblastoid cells, shutdown of RAD51 expression resulted in arrest of majority of cell population in G2/M phase with numerous chromosome breaks, followed by a massive cell death. The persistent G2/M block and release of cells from the block took a long time of about 15h of post treatment in DT 40, RAD52−/− and RAD51D−/− cells, while for XRCC3−/−, XRCC2−/− and RAD51D−/− the was much quicker.

After the first dose of irradiation, a very high percentage of S-phase cells (70–80%) accumulated at about 2–3 hours. It is possible that most of these cells belong to relatively radio resistant phase of the cell cycle. i.e mid and late S-phase. Therefore, the response to the second radiation dose would entirely depend on the surviving cells in these phases and on the repair of the sublethal damage they had sustained from the earlier dose. This is very well reflected with the increase in the survival observed at later intervals of about 2–5 hours of post irradiation.

Finally, both based on our earlier observation and the present study, at least for DT40 chicken B lymphocytes, it can be concluded that although RAD52, XRCC2, XRCC3, RAD51C and RAD51D genes are involved in the DSB repair by homologous recombination in these cells, their role is not as critical as that of RAD54. However, increased radiosensitivity of RAD51C−/− cells, especially at the higher radiation doses indicates its importance in the DSB repair by HR pathway more than its other four counterparts. Moreover, the SLD recovery observed in these cells is a reflection of the repair of DSBs resulting from surviving cells which have sustained the initial damage. The delay in the peak survival could be attributed to the slow progression of the cells through the cell cycle. These findings may apply only to the DT40 cells and may not be necessarily universal.

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