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Title

Increased susceptibility to delayed genetic effects of low dose X-irradiation in DNA repair deficient cells

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Key words

genomic instability, bystander effect, DNA repair, low-dose irradiation

Running title

Genomic instability in repair deficient cells

Abstract

Purpose : To examine whether the levels of micronuclei induction, as a marker for genomic

instability in the progeny of X-irradiated cells, correlates with DNA repair function.

Materials and methods : Two repair deficient cell lines (X-ray repair cross-complementing 1 (XRCC1) deficient cell line (EM9) and X-ray repair cross complementing 5 (XRCC5; Ku80) deficient X-ray sensitive Chinese hamster ovary (CHO) cell line (xrs5)) were used in addition to wild-type CHO cells. These cells were irradiated with low doses of X-rays (up to 1 Gy). Seven days after irradiation, micronuclei formed in binucleated cells were counted. To assess the contribution of the bystander effect micronuclei induction was measured in progeny of non-irradiated cells co-cultured with cells that had been irradiated with 1 Gy.

Results : The delayed induction of micronuclei in 1 Gy-irradiated cells was observed in normal CHO and EM9 but not in xrs5. In the clone analysis, progenies of xrs5 under bystander conditions showed significantly higher levels of micronuclei, while CHO and EM9 did not.

Conclusion : Genomic instability induced by X-irradiation is associated with DSB (double strand break) repair, even at low doses. It is also suggested that bystander signals, which lead to genomic instability, may be enhanced when DSB repair is compromised.

Introduction

Radiation induced genomic instability has been reported by many groups (Kadhim *et al.* 1992; Little *et al.* 1997; Morgan. 2003ab; Suzuki *et al.* 2003, 2009, 2010; Somodi *et al.*

2005; Seoane *et al.* 2007; Toyokuni *et al.* 2009). This form of damage is apparent in growing cells several generations after irradiation of the initial cell population. Such damage can be indicated by abnormal phenotypes, including reproductive cell death, delayed chromosome aberration, gene mutation and transformation. It has been suggested that genomic instability is one of the driving forces in radiation induced carcinogenesis. Some evidence suggests that radiation induced genomic instability has been caused by DNA double strand breaks (DSB) (Chang and Little. 1992; Suzuki *et al.* 2003, 2010). Therefore, understanding the DNA repair mechanisms involved in the expression of genomic instability is important.

There has been significant study of the “radiation-induced bystander effect” as an example of a non-targeted effect of ionizing radiation exposure. This effect is thought to be important for risk estimation of radiation carcinogenesis as it predominantly occurs at low doses (Sawant *et al.* 2001). Not only DNA damage but also epigenetic change is also observed in the bystander effect (Koturbash *et al.* 2007; Ilnytsky and Kovalchuk 2011). Although there are increasing numbers of studies of the bystander response (Hamada *et al.* 2011; Chai and Hei 2008; Prise and O’Sullivan 2009), the mechanisms are not fully understood. It is important to determine the interrelationship between bystander effects and genomic instability.

For DSB, two main mechanisms have been characterized; homologous recombination (HR) and non-homologous end joining (NHEJ). Of these, NHEJ is the major player in the repair

of radiation-induced DNA double strand breaks in mammalian cells (Valerie and Povirk 2003; Goodarzi *et al.* 2010). In this repair process the Ku70/80 (X-ray cross complementing 6/5 (XRCC 6/5)) protein complex stabilizes the ends of digested DNA strands, DNA-Protein Kinase catalytic subunits (DNA-PKcs) become activated on association with the Ku complex, which in turn may activate the enzyme ligase IV with XRCC4 (X-ray cross complementing 4) , Finally, activated ligase IV leads to rejoining reactions in the two ends of DNA (Mahaney *et al.* 2009). From cellular studies, it is well known that a defect of each protein in this process leads to higher cell killing after irradiation because of less repair ability of DNA double strand breaks (Nussenzweig *et al.* 1996). Base damage on DNA caused by irradiation is also well characterized. This damage is repaired by a base excision repair process. XRCC1 is important for activation of ligase III which links the digested strands in this repair process (Caldecott *et al.* 1994; Sterpone and Cozzi 2010). A defect in XRCC1 leads to hypersensitivity to alkylating agents (Shen *et al.* 1998).

In these investigations, repair deficient cell lines derived from Chinese hamster ovary (CHO) cells have been used as described in previous reports (Thacker and Zdzienicka 2003; Kashino *et al.* 2004; Somodi *et al.* 2005). In the present study, we investigated the X-ray induced delayed effects in repair deficient CHO cell lines to elucidate the mechanisms of genomic instability induced by lower doses of X-irradiation.

Materials and Methods

Cell culture

Wild type CHO cells and the Ku80 deficient X-ray sensitive CHO cell line, xrs5, were kindly supplied by Dr Tom K. Hei (Columbia University). EM9 cells, which are an X-ray repair cross-complementing 1 (XRCC1) deficient cell line, were purchased from ATCC (American Type Culture Collection, Manassas, USA). Cells were cultured in Minimum Essential Medium (MEM) alpha medium (Invitrogen Corporation, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Helena Biosciences Europe, Gateshead, UK), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

X-irradiation

Exponentially growing cells in 25 cm² flasks (T25, BD, Franklin Lakes, USA) were irradiated at doses of 0.2, 0.5 or 1 Gy using an X-ray generator (Pantak IV at Gray Cancer Institute, Northwood, UK) operating at 240 kVp and 13 mA with a filter system, composed of a 0.25 mm Cu plus 1 mm Al filter and a 4.3 mm Al flattening filter, at a dose rate of 0.5 Gy / min, or using an X-ray generator (Softex, Tokyo, Japan) at 150 kVp and 5 mA with a 0.1mm Cu filter, at a dose rate of 0.49 Gy/min.

Micronucleus assay

The micronucleus assay used was as described previously (Kashino *et al.* 2004, 2007). Cells were irradiated with 0.2, 0.5, and 1 Gy of X-rays as described above. To investigate acute effects cells were treated with 2 µg/ml cytochalasin B (Wako, Osaka, Japan) for 24 h immediately after X-irradiation. To investigate delayed effects cells were cultured for 7 days after irradiation before treatment with cytochalasin B. Cells were then harvested and treated with 3 ml of hypotonic (0.1 M) KCl (Wako) for 20 min, and then fixed with 3 ml of methanol (Wako) - acetic acid (Wako) (5:1 v/v). The cell suspensions were centrifuged at 145 g for 5 min, the supernatant was discarded and cells were re-suspended in 4 ml methanol-acetic acid solution and incubated on ice for 5 min. Following re-centrifugation, the supernatant was discarded and 0.5-1 ml methanol-acetic acid solution was added. Cells were suspended and dropped onto glass slides (Matsunami Glass, Osaka, Japan). The slides were stained with 7.5% Giemsa (Merck chemical, Tokyo, Japan) for 40 min. Percentage of binucleated cells with micronuclei were counted microscopically as described previously (Fench *et al.* 2003). The yields of micronuclei per 6000 binucleated cells in three independent experiments were scored in each group.

Clonal analysis of delayed effect by co-culture method

Clonal analyses for delayed effects in directly irradiated and bystander conditions were performed using a co-culture method in CHO, EM-9 and xrs5 cells. To distinguish the

irradiated cells and non-irradiated cells in co-culture, we used hypoxanthine phosphoribosyltransferase (*HPRT*) mutant and normal cells (*HPRT* is wild-type). *HPRT* mutant cells were obtained spontaneously after 60 μ M 6-thioguanine (6TG, Tokyo Kasei, Tokyo, Japan) selection. In co-culture of irradiated cells with non-irradiated cells, 5×10^5 of *HPRT* mutant cells were irradiated with 1 Gy of X-rays, and these cells were transferred to T25 flasks with the same number of non-irradiated *HPRT* normal cells. We regarded this co-culture equivalent to the bystander condition. In contrast to the bystander condition, non-irradiated mutant and normal *HPRT* cells were co-cultured in the same T25 (control), and irradiated mutant and normal *HPRT* cells were co-cultured in the same T25 (irradiated condition). For these three conditions, cells were cultured for 7 days with the transfer of cells into a new T25 every 2 or 3 days. 100 cells from the co-cultured population in T25 were transferred into a 100 mm petri dish with hypoxanthine-aminopterin-thymidine (HAT, Sigma-Aldrich, Tokyo, Japan) medium and incubated for 7-10 days to make some colonies HAT resistant. HAT resistant cells are *HPRT* normal cells, because *HPRT* mutant cells cannot survive in the HAT medium. Colonies were selected at random for further analysis for the progenies of non-irradiated cells, bystander cells and irradiated cells. Cells chosen were expanded to $2 - 5 \times 10^6$ and the micronucleus assay performed as described above.

Statistical analysis

Statistical analysis was performed using Student's *t* test and one-way analysis of variance (ANOVA).

Results

The results of micronuclei induction immediately after irradiation are shown in Figure

1. In all dose points examined, the yields of micronuclei in xrs5 cells were significantly higher than in the control CHO cells ($p < 0.001$ by student *t* test), and those in EM9 cells were slightly higher. These results are consistent with differences in clonogenic survival (data not shown), and show that micronuclei formed immediately after irradiation are mainly caused by abnormal functions in the repair of DNA double strand breaks. The results of delayed micronuclei formations are shown in Figure 2. Dose dependent increases were observed in CHO and EM9 cells up to 1 Gy, additionally, significantly higher levels of micronuclei were observed in both CHO and EM9 after 0.5 or 1 Gy irradiation compared to non-irradiated cells. Interestingly, no delayed inductions of micronuclei, above control levels, were observed in xrs5 cells at these doses. The percentage of binucleated cells with micronuclei in xrs5 cells one week after irradiation of 0 to 1 Gy were from 9.6 to 9.9%. As we could not exclude the possibility that cytogenetic damage by direct irradiation are included in the results in Figure 2, delayed effects in the irradiated population were examined in clones of irradiated cells as described in Figure 3.

In order to clarify the contribution of a bystander effect in the delayed response, cells receiving bystander signals from irradiated cells via co-culture were also examined, using an *HPRT* mutation selection process (Figure 3). Six to nine clonal populations for each cell line (non-irradiated, bystander, irradiated) were analyzed. As shown in Figure 4, some progenies of irradiated CHO and EM9 showed higher levels of micronuclei, but not *xrs5*. Average percentage of binucleated cells with micronuclei are shown as bars in Figure 4, and significant differences were observed between the non-irradiated and irradiated condition in CHO ($p<0.05$). Although increased levels of bystander induced micronuclei were not observed in CHO or EM9, *xrs5* showed significantly higher levels.

Discussion

In the present study, we confirmed the induction of delayed genetic effects especially at low X-ray doses. As shown in our previous report (Somodi *et al.* 2005), cells that are defective in single strand breaks repair are susceptible to radiation induced delayed effects at high doses. Our present study also shows higher delayed induction of micronuclei in EM9, suggesting that unrepaired or misrepaired DNA damage are important for the expression of delayed genetic effects at low X-ray doses. In directly irradiated Ku80 deficient *xrs5* cells, no delayed induction of micronuclei is observed at 7 days post-irradiation and about 3 weeks in the

case of clonal analysis, implicating a Ku80 dependent process in the induction of delayed effect. Although we could not exclude the possibility that delayed effects are induced much later time in *xrs5*, this result is supported by previous reports (Chang and Little 1992; Suzuki *et al.* 2010) which suggest that non-homologous end joining (NHEJ) occurs immediately after irradiation, leading to mis-rejoining sites, which are the main trigger of genomic instability. However, higher levels of micronuclei in progenies of bystander cells were detected in our experiment using Ku80 deficient *xrs5* (Figure 4). Therefore, not only NHEJ but also another process, that is characteristically induced by bystander effect in DSB repair deficient cells, is involved in delayed effects. In the report by Watson *et al.* (2000), the contribution of bystander effects to genomic instability was suggested by the analysis of chromosome aberration in mouse bone marrow cells. Our data strongly support this idea.

We need to clarify why only bystander but, not directly irradiated cells, showed delayed effects in the *xrs5* line (Figure 4). We have previously reported higher induction of micronuclei via a bystander effect in *xrs5* compared to CHO and EM9 (Kashino *et al.* 2004; Kashino *et al.* 2007). However, increased levels of micronuclei induced by the bystander effect in previous reports are much less than those in directly irradiated cells. Therefore, bystander induced DSBs are unlikely to be a trigger of delayed micronuclei induction in *xrs5* in the present study. Also, we have hypothesised that mitochondrial modulation is involved in

genomic instability by bystander effect. Our recent results suggest that mitochondrial membrane potentials were similarly reduced both in directly irradiated and bystander cells in the range from 0.2 to 4 Gy (data not shown). This epigenetic mitochondrial modulation seems to lead to the production of ROS (Kobashigawa *et al.* 2011), which is a key factor in genomic instability (Clutton *et al.* 1996, Roy *et al.* 2000, Limoli *et al.* 2003, Rugo *et al.* 2004, Tominaga *et al.* 2004, Slane *et al.* 2006). Due to the higher sensitivity of *xrs5*, unrepaired irradiated cells, in which mitochondrial modulation has occurred, should be removed from the population during clonal expansion. In *xrs5* bystander cells, lethal effects are increased, but the level for mitochondrial modulation is sufficiently reduced. Therefore, it is possible that for *xrs5* bystander cells, cell survival and genomic instability is maintained through reactive oxygen species (ROS) production by mitochondrial modulation. In bystander conditions, CHO and EM9 clones did not show higher levels of micronuclei, therefore, a Ku80 deficient phenotype is involved in genomic instability through a bystander dependent mechanism. We aim to clarify the epigenetic phenotype leading to genomic instability in future studies.

In conclusion, this study highlights that DSB repair deficiency leads to the accumulations of unstable progeny cells thorough bystander effects, while the DSB repair process contributes to genomic instability via micronucleus induction in the progeny of directly irradiated cells.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Figure 1 Micronuclei induction immediately after X-irradiation in repair deficient CHO cell lines (the acute effect). The average yields of binucleated cells with micronuclei $\pm$ standard error of mean (SEM) from three individual experiments are shown for control CHO (closed circle), repair deficient EM-9 (closed triangle) and xrs5 (open triangle).

Figure 2 Micronuclei induction one week after X-irradiation in repair deficient CHO cell lines (delayed effect). The average yields of binucleated cells with micronuclei $\pm$ standard error of the mean (SEM) from three individual experiments are shown for control CHO (upper panel), repair deficient EM9 (middle panel) and xrs5 (lower panel) cells. Significant differences were observed between non-irradiated cells and irradiated cells (* $p < 0.05$ by student t test).

Figure 3 A schema of clone analysis in non-irradiated, bystander and irradiated conditions.

HAT^R and HAT^S means HAT resistant (HPRT normal cells) and HAT sensitive (HPRT mutant cells), respectively. In the non-irradiated condition (upper), bystander condition (middle) and irradiated condition (lower), HAT resistant clones were obtained after HAT selection, and were analyzed .

Figure 4 Percentage of micronuclei in clonal analysis. The levels of micronuclei in each clone were analyzed in the control non-irradiated condition (left), bystander condition (middle) and irradiated condition (right). Each plot represents the result in each clonal population obtained from each condition as described in Materials and Methods. Bars in the figure show the average for each condition. Significant differences were observed between the distribution of control and bystander or irradiated condition (* $p < 0.05$ by one-way ANOVA).

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