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Evidence for induction of DNA double strand breaks in the bystander response to targeted soft X-rays in CHO cells

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Abstract

This study investigated the role of DNA double strand breaks and DNA base damage in radiation-induced bystander responses in Chinese hamster ovary (CHO) cell lines. Two CHO repair-deficient clones, xrs5 (DNA double strand break repair-deficient) and EM9 (DNA base excision repair-deficient) were used in addition to the wild type (CHO). The Gray Cancer Institute ultrasoft X-ray microprobe is a powerful tool for investigating the bystander response, because it permits the irradiation of only a single nucleus of a cell, as reported previously. In order to investigate the bystander effect in each repair-deficient cell line, we irradiated a single cell within a population and scored the formation of micronuclei. When a single nucleus in the population was targeted with 1 Gy, elevated numbers of micronuclei were induced in the neighbouring unirradiated cells in the EM9 and xrs5 cell lines, whereas induction was not observed in CHO. The induction of micronuclei in xrs5 was significantly higher than that in EM9. Under these conditions, the surviving fraction in the neighbouring cells was significantly lower in xrs5 than in the other cell lines, showing a higher cell killing effect in xrs5. To confirm that bystander factors secreted from irradiated cells caused these effects, we carried out medium transfer experiments using conventional X-irradiation. Medium conditioned for 24 h with irradiated cells was transferred to unirradiated cells and elevated induction of micronuclei was observed in xrs5. These results suggest that DNA double strand breaks rather than base damage are caused by factors secreted in the medium from irradiated cells.

Keywords: Bystander effect; DNA repair; DNA double strand break; Base damage; Soft X-ray; Microbeam

1. Introduction

It is thought that damage signals may be transmitted from irradiated to unirradiated cells in a population, leading to a variety of genetic effects via a bystander

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effect. It has been reported that the bystander effect can be mediated via gap junction intercellular communication [1–3] and also factors secreted from irradiated cells via the culture medium in vitro [3–6]. As it has been reported that bystander cells show a variety of cellular effects which result in cell death and chromosome aberrations, DNA damage should be observed in a radiation-induced bystander response [1–6]. However, it is still not clear which type of DNA damage is responsible for bystander effects.

It is suggested that the bystander effect induced by ionizing radiation might lead to carcinogenesis. In vitro mutation assays have been useful for the estimation of the risk of carcinogenesis. Little et al. have reported that the mutation frequency at hprt locus is increased in bystander Chinese hamster ovary (CHO) cells which are exposed to very low fluences of α particles [7]. Zhou et al. showed a higher frequency of gene mutations in bystander cells at the CD59 locus in a hybrid hamster cell line by using alpha particle microbeam irradiation [2]. However, PCR analysis by Zhou et al. showed that a higher yields of deletion mutations were induced by microbeam irradiation in bystander cells, whereas most mutations analyzed by Little et al. were suggested to be mainly point mutations in bystander cells [2,7]. Thus, these two reports are in disagreement regarding the type of mutations produced in bystander cells. Investigations are necessary to resolve the type of DNA damage resulting from the radiation-induced bystander effect.

DNA damage is repaired by several efficient processes within cells. For the repair of DNA double strand breaks, molecular studies have elucidated two main pathways after direct irradiation of cells [8]. Nonhomologous end joining (NHEJ) is the main repair pathways for DNA double strand breaks [9]. In this repair process the Ku70/80 protein complex stabilizes the ends of the fragmented DNA strands and the DNA-PK catalytic subunit (DNA-PKcs), which may activate ligase IV with XRCC4, is activated by association with Ku complex. Finally, activated ligase IV leads to rejoining reactions in the two ends of the DNA. It is well known that a defect of any protein in this process leads to higher cell killing effect after irradiation because of less repair ability of DNA double strand breaks [10–15]. On the other hand, DNA base damage is also induced by irradiation and many of these are associated with clustered damage formed at the sites of individual tracks crossing the DNA [16]. Base damage is repaired by base excision repair processes, where XRCC1 is important for the activation of ligase III which links the digested strands in this repair process [17,18]. A defect in XRCC1 leads to hypersensitivity to alkyating agents [19]. These repair mechanisms recognize a specific damage immediately after irradiation and remove it. Therefore, it is difficult to detect the exact level of each specific type of DNA damage induced by radiation, especially at low doses. DNA repair-deficient Chinese hamster ovary (CHO) cell lines, which are deficient in Ku80 and XRCC1, have been used to detect DNA damage efficiently, and greatly facilitates the detection of small numbers of DNA damages as described in a previous report [14].

Microbeams are useful for investigation of bystander responses. The ultrasoft X-ray microprobe at Gray Cancer Institute is the first microbeam facility to use X-rays for radiobiological purposes, and is able to irradiate a single cell with beams focused down to micrometer accuracy [20–24]. We reported previously that irradiating a single V79 cell with X-rays leads to bystander cell killing in about 10% of the cells in the population [24]. The study of the bystander effect using this soft X-ray microbeam is expected to be applicable to not only to survival assays but also to other biological endpoints used for detection of chromosome damage, such as the in vitro micronucleus assay, as this assay has already been used in bystander studies utilising charged particle microbeams [6].

The present results indicate that the bystander effect in DNA double strand break repair-deficient xrs5 cells is significantly higher than that in base excision repair-deficient EM9 cells and control CHO cells, suggesting that DNA double strand breaks are the principal form of damage observed in unirradiated bystander cells.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary (CHO) cells and xrs5 cells were kindly supplied by Dr. Tom K. Hei, Columbia University, New York, and EM9 cells were purchased from ATCC (American Type Culture Collection, VA, USA). Cells were cultured in MEM alpha medium (Invitrogen Ltd., Paisley, UK) supplemented with 10%

FBS (Helena Biosciences Europe), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen Ltd., Paisley, UK). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Micronucleus assay

To investigate the induction of micronuclei by direct X-irradiation, the cells were irradiated with 0.2, 0.5, and 1 Gy of conventional X-rays. Exponentially growing cells in T25 flasks were irradiated with X-rays using an X-ray generator (Pantak IV) operating at 240 kVp and 13 mA with a filter system composed of 0.25 mm Cu plus 1 mm Al filter and 4.3 mm Al flattening filter, at a dose rate of 0.5 Gy/min. Either immediately after irradiation or following 24 h incubation, cells were treated with 2 μg/ml cytochalasin B for 24 h in a T25 flask. They were then harvested and treated with 3 ml of hypotonic (0.1 M) KCl for 20 min, and fixed with 3 ml of methanol–acetic acid (5:1). The cell suspensions were centrifuged at 1200 rpm for 5 min, the supernatant removed and cells resuspended in 4 ml methanol–acetic acid solution and incubated on ice for 5 min. After further centrifugation, the supernatant was removed and 0.5–1 ml methanol–acetic acid solution was added. Cells were resuspended and a sample was dropped onto slides and stained with 7.5% Giemsa for 40 min. Micronuclei per 2000 binucleated cells were counted.

To investigate the bystander effect, localised irradiation was carried out using the Gray Cancer Institute focused ultrasoft X-ray microprobe. The procedure has been described in detail elsewhere [20,24]. Briefly, the day before, the experiment cells (5×10^4) were seeded on 0.9 μm-thick Mylar film (Goodfellow Ltd., UK). Cells were stained with 100 nM Hoechst 33342 for 1 h prior to irradiation. After removal of stain, an area around the centre of the dish was scanned in order to identify a precise single nucleus. A single cell was irradiated with 1 Gy of aluminum or carbon K-shell characteristic X-rays (Al_k = 1.49 keV or C_k = 0.28 keV) produced by a focused ultrasoft X-ray microprobe.

The X-ray microbeam targeted a single cell at a dose rate of 0.1 Gy/s. The medium was changed and cells were incubated with cytochalasin B for 24 h either immediately after irradiation or 24 h later. Slides were prepared as described above.

2.3. Survival assay

The surviving fraction was determined by a clonogenic survival assay. Individual cells, stained with 100 nM Hoechst 33342, were scanned using the Gray Cancer Institute X-ray microprobe system, as described previously [20,24]. After 100–200 cells were scanned, a single cell was irradiated with 1 Gy of Al_k or C_k produced by a focused ultrasoft X-ray microprobe. Cells were incubated for 4 days, stained with 100 nM Hoechst 33342, and the dishes scanned to revisit the original locations and test for the presence of colonies. Control cells were scanned, without irradiation under the same conditions, and surviving fractions were calculated.

2.4. Medium transfer experiment

Cells (5×10^4) were seeded onto six-well plates one day prior to irradiation. Immediately before irradiation, medium was changed and cells were irradiated with 1 Gy of conventional X-rays. Cells were incubated for 24 h following irradiation. The culture medium was filtered through a 0.22 μm filter and transferred to unirradiated cultured cells on six-well plates. Cytochalasin B was added at the same time as the medium transfer, and cells were incubated for 24 h. Micronucleus samples were prepared as described above.

2.5. Statistical analysis

The statistical analysis in the present study was performed using Student's *t*-test.

3. Results

3.1. Direct effect of X-rays on repair-deficient cells

The sensitivity to direct irradiation by low-dose X-rays in repair-deficient CHO cell lines were examined using the micronucleus assay. As shown in Fig. 1, EM9 cells were slightly more sensitive than CHO, whereas xrs5 cells were significantly more sensitive ($p < 0.001$). Following 1 Gy-irradiation the yield of micronuclei per 2000 binucleated cells in CHO, EM9 and xrs5 were 224, 465 and 1287, respectively (Fig. 1). Induction of micronuclei in xrs5 cells was also detectable in this

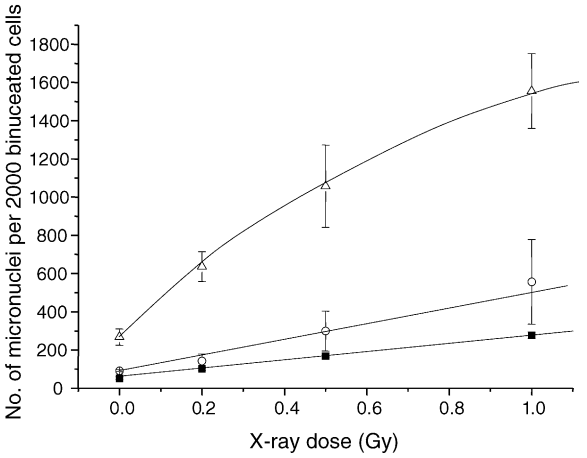

Fig. 1. Micronuclei induced by conventional X-irradiation in all cells of CHO (square), EM9 (circle) and xrs5 (triangle). Cells were incubated with cytochalasin B for 24 h immediately after irradiation. Results are means ± S.D. from three separate experiments.

assay when cells were irradiated at 0.05 Gy of X-ray (data not shown). However, inductions of micronuclei in EM9 cells were not detectable at that dose. Therefore, direct irradiation with low-dose X-rays may cause micronuclei formation through unrepaired or misrepaired DNA double strand breaks in xrs5.

3.2. Bystander effects using X-ray microbeams in CHO repair-deficient cells

The results of the bystander effect in the micronucleus assay are shown in Fig. 2. Two conditions, where cytochalasin B was added at different times, were examined in the present study. The left and right panels in Fig. 2 show the results following a total incubation time of 24 and 48 h respectively, after 1 Gy irradiation with the X-ray microbeam. The yields of micronuclei in CHO cultures with or without a single irradiated cell were between 75 and 84 micronuclei per 2000 binucleated cells. This shows no bystander effect under these conditions. In EM9 cells 19 micronuclei per 2000 binucleated cells were induced by a single cell soft-X-ray irradiation in both 24 and 48 h incubated samples (Figs. 2 and 3), suggesting micronuclei were induced through a bystander response in unirradiated neighbour cells. With xrs5, 40 and 74 micronuclei per 2000 binucleated cells were induced by X-ray microbeam irradiation in the 24 and 48 h incubated samples, respectively (Figs. 2 and 3). Therefore, the bystander effect observed in xrs5 was higher than that in EM9. Also, the induced level of micronuclei in xrs5 was increased further during the 24 h incubation following microbeam irradiation (Fig. 3).

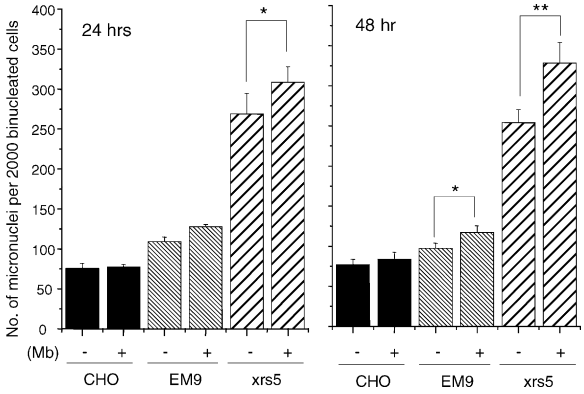

Fig. 2. Micronuclei induced by X-ray microbeam irradiation of a single cell in the population of CHO, EM9 and xrs5. Twenty-four (left panel) and 48 h (right panel) after irradiation, micronuclei samples were prepared. During the last 24 h incubation, cells were incubated with cytochalasin B. Results are mean micronuclei per 2000 binucleated cells ± S.E.M determined from three (24 h incubation) or four (48 h incubation) independent experiments. 'Mb + and −' indicates whether a single cell in the population was irradiated or not. Significant differences were observed in EM9 and xrs5 (Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).

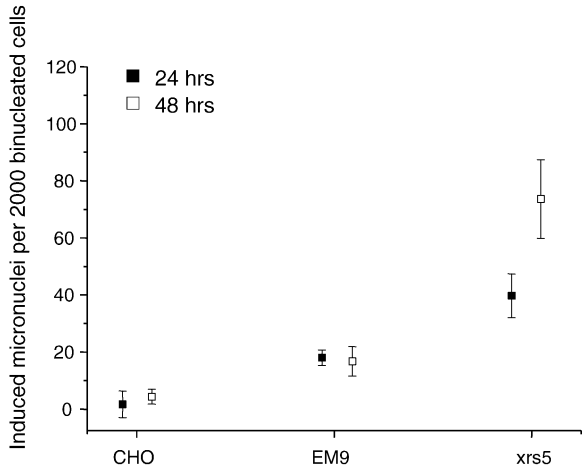

Fig. 3. Micronuclei induced per 2000 binucleated cells by microbeam irradiation. Total incidence minus the background level of micronuclei is represented at 24 h (solid symbols) and 48 h (open symbols). The data were obtained using the data from Fig. 2.

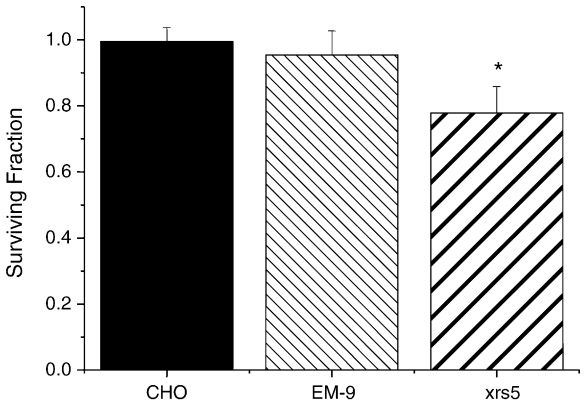

Fig. 4. The clonogenic surviving fractions in CHO, EM9 and xrs5 cells. A single cell was irradiated with 1 Gy of X-ray microbeam. Results are the means ± S.E.M from three independent experiments. A significant difference was observed between the surviving fractions in CHO and xrs5 (Student's *t*-test, $^{*}p < 0.05$).

The results of the clonogenic survival assay are shown in Fig. 4. The surviving fraction in the cell population of CHO and EM9 was not affected when a single cell in the population was irradiated with the X-ray microbeam (surviving fractions in CHO and EM9 were 0.99 and 0.95, respectively). However, a significant cell killing effect was observed in xrs5 cells (surviving fraction was 0.78). These results suggest that a defect in DNA double strand break repair leads to increased cell killing in unirradiated cells through a bystander response.

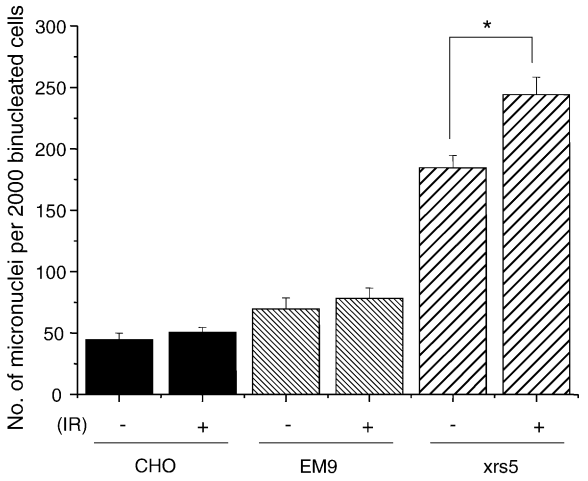

Fig. 5. Micronuclei induced by the transfer of conditioned medium in CHO, EM9 and xrs5 cells. 'IR + and −' indicates whether the conditioned medium transferred to unirradiated cells, had been irradiated in the presence or absence of cells, respectively. Cells were incubated for 24 h, after irradiation before the conditioned medium was transferred to unirradiated cells. Results show mean numbers of micronuclei ± S.E.M per 2000 binucleated cells from three independent experiments. A significant difference was observed between irradiated and unirradiated conditions in xrs5 (Student's *t*-test, $^{*}p < 0.05$).

3.3. Media transfer experiments

Cells were irradiated with 1 Gy of conventional X-rays and incubated for 24 h. The medium was then transferred to an unirradiated cell population. The result showed that medium which had been conditioned by incubation with irradiated cells induced significant numbers of micronuclei in unirradiated xrs5 cells (Fig. 5). The average number of induced micronuclei by conditioned medium measured in CHO, EM9 and xrs5 were 6.0, 8.7 and 59.7 per 2000 binucleated cells, respectively (Fig. 5). A higher induction of micronuclei was also observed in unirradiated xrs5 cells following transfer of conditioned medium from irradiated CHO cells (data not shown).

4. Discussion

In the present study, we have shown that the radiation-induced bystander effects are much higher in DNA double strand break repair -deficient cells than in base damage (and single strand break) repair-deficient cells. Although further mechanistic studies are needed, this result suggests that mainly DNA double strand breaks rather than base damage are induced in bystander cells. In the microbeam experiments cells were seeded at low density, consequently there was no direct cell-to-cell contact with others in the population. In this situation any bystander effect should be caused by diffusion of factors, through the medium, from an irradiated cell. This was confirmed by the medium transfer experiments which showed higher bystander effects in xrs5 cells (Fig. 5).

The bystander effect was not observed when only medium was irradiated with X-rays from the microbeam (data not shown). The medium transfer experiment showed that the conditioned medium from irradiated CHO-K1 cells (normal repair ability) also induced micronuclei significantly in unirradiated xrs5

cells (data not shown). These results suggest that the 'secretion level' of the bystander factor for cell killing and micronucleus formation does not depend on the repair capacity of irradiated cells. However the level of bystander factors secreted from irradiated cells should depend on the type of cells irradiated as suggested by Mothersill et al. [4]. Moreover, it seems that the bystander effect occurs continuously during the incubation period of an irradiated cell with unirradiated cells, because the number of induced micronuclei found 48 h after microbeam irradiation is higher than that after 24 h incubation in xrs5, as shown in Figs. 2 and 3. We concluded that the soluble factor secreted from irradiated cells, even when a single cell was targeted with soft-X-rays, reacts with unirradiated cells continuously, and the sensitivity to the factor depends on the DNA repair proficiency in the bystander cells.

The results presented here showing a higher degree of bystander effect in xrs5 cells are consistent with the two previous reports by Nagasawa et al. [25,26] which show bystander effects in the chromosome aberration assay and mutation assay. The repair-deficient clone xrs5 has a defect in the function of Ku80 and the bystander effect observed may be related to this. It has been suggested that the lower repair ability of DNA double strand breaks in the bystander cells leads to chromosome aberrations or deletion mutations through unrepaired and misrepaired DNA double strand breaks, and should be linked to a higher yield of micronuclei and a lower surviving fraction. EM9 cells, which have reduced base excision repair capacity, also showed higher micronuclei induction compared to control CHO cells after microbeam irradiation (Figs. 2 and 3). As EM9 cells have shown higher sensitivity to direct X-irradiation in the micronucleus assay (Fig. 1), it is proposed that misrepair of delayed repairability of base damage or single strand breaks also lead to the formation of micronuclei. It is likely that some clustered damage, such as base damage from oxidative damage or single strand breaks, form DNA double strand breaks, as reported previously [27]. However, the yields of micronuclei induced by the bystander response in EM9 was much smaller than that in xrs5, implying that DNA double strand breaks are induced in bystander cells independently of base damage or single strand breaks.

In a previous study, in which a single cell was irradiated in V79 cell population a bystander response measured as 10% cell killing was detected [24]. This effect was not observed in the wild-type CHO cells used in the present study (Fig. 4), only in the dsb-repair-deficient mutant. Although further work is required, differences in the relative repair capacity of these two cell lines and the influence of cell cycle sensitivity to bystander responses may play a role (Schettino et al., unpublished).

Although the detail is still not clear, we can divide the mechanism of bystander induction of DNA double strand breaks through the culture medium into three steps. Firstly the bystander factor is secreted from irradiated cells. Short-lived radicals such as ROS and NO are induced after irradiation, and may be implicated in this process, as radical scavengers can be effective for suppression of bystander response [3,5,6]. The second step is the diffusion of the bystander factors from irradiated cells through the culture medium. To have any effect, these short-lived radicals must be converted to long-lived radicals such as the radical reported recently to cause mutations indirectly in the cells [28–30], or cytokines such as TGF-β1 which has been reported to be involved in radiation-induced bystander responses [31,32]. Finally the signal transduction pathways (from the surface to the inside of cell) must be activated in bystander cells. Our present results suggest that DNA double strand breaks are induced in bystander cells, therefore an oxidative stress-independent signal pathway should be activated in bystander cells. As chromosome damage inducing micronuclei are caused by the alteration of stable conformation of chromatin structure, one possibility is that the activation of chromatin remodeling, which is susceptible to DNA double strand breaks, is induced in bystander cells. Further investigations are required to define the bystander factors and the related signal transduction processes necessary for the bystander response.

Acknowledgements

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