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Evidence for Induction of DNA Double Strand Breaks in the Bystander Response to Targeted Soft X-Rays in Repair Deficient CHO Cells

Genro KASHINO,[1] Keiji SUZUKI,[1] Kevin M. PRISE,[2] Giuseppe SCHETTINO,[2] Melvyn FOLKARD,[2] Borivoj VOJNOVIC,[2] Barry D. MICHAEL,[2] Seiji KODAMA,[3] Naoki MATSUDA,[4] Masami WATANABE[5]

[1] Division of Radiation Biology, Department of Radiology and Radiation Biology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[2] Gray Cancer Institute, Middlesex, UK

[3] Radiation Biology Laboratory, Radiation Research Center, Frontier Science Innovation Center, Organization for University-Industry-Government Cooperation, Osaka Prefecture University, Sakai, Japan

[4] Division of Radiation Biology and Protection, Center for Frontier Life Sciences, Nagasaki University, Nagasaki, Japan

[5] Division of Radiation Life Sciences, Research Reactor Institute, Kyoto University, Kumatori, Japan

Evidence is accumulating that irradiated cells produce some signals which interact with non-exposed cells in the same population. Here, we analysed the mechanism of such a bystander effect from targeted cells to non-targeted cells. Firstly, in order to investigate the bystander effect in CHO cell lines we irradiated a single cell within a population and scored the formation of micronuclei. When a single nucleus in the population, of double strand break repair deficient xrs5 cells, was targeted with 1Gy of Al-K soft X-rays, elevated numbers of micronuclei were induced in the neighbouring unirradiated cells. The induction of micronuclei was also observed when conditioned medium was transferred from irradiated to non-irradiated xrs5 cells. These results suggest that DNA double strand breaks are caused by factors secreted in the medium from irradiated cells. To clarify the involvements of radical species in the bystander response, cells were treated with 0.5%DMSO 1 hour before irradiation and then bystander effects were estimated in xrs5 cells. The results showed clearly that DMSO treatment during X-irradiation suppress the induction of micronuclei in bystander xrs5 cells, when conditioned medium was transferred from irradiated xrs5 cells. Therefore, it is suggested that radical species induced by ionizing radiation are important for producing bystander signals.

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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 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]

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.[7] Non-homologous end joining (NHEJ) is the main repair pathways for DNA double strand breaks.[8] 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.[9-14] 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.[15] 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

Address correspondence: Genro Kashino, Ph.D., Division of Radiation Biology, Department of Radiology and Radiation Biology, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521 JAPAN
TEL: +81-(0)95-819-2460, FAX: +81-(0)95-819-2460, E-mail: kashino@net2.nagasaki-u.ac.jp

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process.[16,17] A defect in XRCC1 leads to hypersensitivity to alkyating agents.[18] 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.[13]

We can divide the mechanism of bystander response into three steps. Firstly the bystander factor is secreted from irradiated cells. It is thought that some of signal activation causing by ionizing radiation should be the trigger of bystander response. The second step is the diffusion of the bystander factors from irradiated cells through the culture medium. There are some evidences that some cytokines are candidate for bystander factor(s). Finally the signal transduction pathways (from surface to inside of cell) must be activated in bystander cells. However, these mechanisms are not fully understood. In the present study, we tried to understand the biological effect of bystander effects in non-targeted cells and to clarify the involvement of radical species on producing bystander signal in targeted cells.

Materials and Methods

Cell culture

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 parental CHO line. 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 37ºC in a humidified atmosphere with 5% CO_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 1,200 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.[19,20] 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/sec. The medium was changed and cells were incubated with cytochalasin B for 24 hrs either immediately after irradiation or 24 h later. Slides were prepared as described above.

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.[19,20] 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.

Medium transfer experiment

Cells (5×10^4) were seeded onto 6 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 hrs following irradiation. The culture medium was filtered through a 0.22 μm filter and transferred to unirradiated cultured cells on 6 well plates. Cytochalasin B was added at the same time as the medium transfer, and cells were incubated for 24 hrs. Micronucleus samples were prepared as described above.

Statistical analysis

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

Results and Discussion

The sensitivity to direct irradiation by low dose X-rays in repair deficient CHO cell lines were examined using the micronucleus assay.

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.

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 hr incubated samples, 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 hr incubated samples, respectively. Therefore, the bystander effect observed in xrs5 was higher than that in other cell lines. Also, the induced level of micronuclei in xrs5 was increased further during the 24 hr incubation following microbeam irradiation.

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.

In the media transfer experiments, cells were irradiated with 1 Gy of conventional X-rays and incubated for 24 hrs. 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. Moreover, when irradiated cells were treated with 0.5% DMSO 1hr before irradiation, micronuclei induction was not observed in conditioned medium treated xrs5 cells. This suppressing effect of bystander response by DMSO was observed only when irradiated cells were treated with DMSO, suggesting that radical species scavenged by DMSO are the candidate for the trigger of bystander response. Reactive oxygen species (ROS) or NO (Nitric oxide) induced by ionizing radiation should be activator for the signal transduction pathway leading to the secretion of bystander factor(s) in targeted cells.

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References

1. Azzam EI, de Toledo SM, Gooding T, Little JB. Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. *Radiat Res* 150: 497-504, 1998
2. Zhou Z, Randers-Pehrson G, Waldren CA et al. Induction of a bystander mutagenic effect of alpha particles in mammalian cells. *Proc Natl Acad Sci USA* 97: 2099-2104, 2000
3. Shao C, Furusawa Y, Aoki M et al. Role of gap junctional intercellular communication in radiation-induced bystander effects in human fibroblasts. *Radiat Res* 160: 318-323, 2003
4. Mothersill C, Seymour C. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *Int J Radiat Biol* 71: 421-427, 1997
5. Shao C, Furusawa Y, Aoki M et al. Nitric oxide-mediated bystander effect induced by heavy-ions in human salivary gland tumour cells. *Int J Radiat Biol* 78: 837-844, 2002
6. Shao C, Stewart V, Folkard M et al. Nitric oxide-mediated signaling in the bystander response of individually targeted glioma cells. *Cancer Res* 63: 8437-8442, 2003
7. Bedford JS, Dewey WC. Radiation Research Society. 1952-2002, Historical and current highlights in radiation biology: has anything important been learned by irradiating cells? *Radiat Res* 158: 251-291, 2002
8. Valerie K, Povirk LF. Regulation and mechanisms of mammalian double-strand break repair. *Oncogene* 22: 5792-5812, 2003
9. Gu Y, Seidl KJ, Rathbun GA et al. Growth retardation and leaky SCID phenotype of Ku70-deficient mice. *Immunity* 7: 653-665, 1997
10. Nussenzweig A, Sokol K, Burgman P et al. Hypersensitivity of Ku80-deficient cell lines and mice to DNA damage: the effects of ionizing radiation on growth, survival, and development. *Proc Natl Sci Acad USA* 94: 13588-13593, 1997
11. Fulop GM, Phillips RA. The scid mutation in mice causes a general defect in DNA repair. *Nature* 343: 479-482, 1990
12. Delacote F, Han M, Stamato TD et al. An xrcc4 defect or wortmannin stimulates homologous recombination specifically induced by double-strand breaks in mammalian cells. *Nucleic Acids Res* 30: 3454-3463, 2002
13. Thacker J, Zdzienicka MZ. The mammalian XRCC genes: their roles in DNA repair and genetic stability. *DNA Repair* 2: 655-672, 2003
14. Grawunder U, Zimmer D, Fugmann S et al. DNA ligase IV is essential for V(D)J recombination and DNA double-strand break repair in human precursor lymphocytes. *Mol Cell* 2: 477-484, 1998
15. Rydberg B. Radiation-induced DNA damage and chromatin structure. *Acta Oncol* 40: 682-685, 2001
16. Caldecott KW, McKeown CK, Tucker JD et al. An interaction between the mammalian DNA repair protein XRCC1 and DNA ligase III. *Mol Cell Biol* 14: 68-76, 1994
17. Cappelli E, Taylor R, Cevasco M et al. Involvement of XRCC1 and DNA ligase III gene products in DNA base excision repair. *J Biol Chem* 272: 23970-23975, 1997
18. Shen MR, Zdzienicka MZ, Mohrenweiser H et al. Mutations in hamster single-strand break repair gene XRCC1 causing defective DNA repair. *Nucleic Acids Res* 26: 1032-1037, 1998
19. Folkard M, Schettino G, Vojnovic B et al. A focused ultrasoft X-ray microbeam for targeting cells individually with submicrometer accuracy. *Radiat Res* 156: 796-804, 2001
20. Schettino G, Folkard M, Prise KM et al. Low-dose studies of bystander cell killing with targeted soft X rays. *Radiat Res* 160: 505-511, 2003