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Different involvement of radical species in irradiated and bystander cells

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Abstract

Purpose: To examine whether nitric oxide (NO) and other radical species are involved in radiation-induced bystander effects in normal human fibroblasts.

Materials and methods: Bystander effects were modeled by co-culture of non-irradiated cells with X-irradiated cells, and induction levels of micronuclei in co-cultured non-irradiated cells were examined. Three types of radical scavenger, 2-(4-carboxyphenyl)-4,4,5,5- tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), dimethylsulfoxide (DMSO) and ascorbic acid phosphoric ester magnesium salt (APM), were used to discover which types of radicals are involved in bystander responses.

Results: When irradiated cells were treated with c-PTIO, known to be an NO scavenger, the induction of micronuclei in non-irradiated bystander cells was suppressed. On the other hand, bystander effects were most effectively suppressed when non-irradiated bystander cells were treated with ascorbic acid, known to be a scavenger of long lived radicals.

Conclusion: These results suggest that NO participates in bystander signal formation in irradiated cells but not in bystander cells that are receiving bystander signals.

Keywords: *Bystander effect, nitric oxide (NO), reactive oxygen species (ROS)*

Introduction

It has recently been shown that non-irradiated cells are affected by signals from irradiated cells (Nagasawa & Little 1992, Mothersill & Seymour 1997, Prise et al. 1998, Zhou et al. 2000, Kashino et al. 2004). This so-called bystander effect is thought to be important for risk estimation of radiation carcinogenesis as it predominantly occurs at low doses (Sawant et al. 2001). Many reports concerning bystander effects have been published in recent years (Hamada et al. 2007), but the mechanisms are not fully understood. It is important to determine the trigger for the formation of bystander response; at present it is unclear how this process is initiated. As some papers have suggested that reactive oxygen species (ROS) and nitric oxide (NO) are involved in the bystander response (Iyer et al. 2000, Matsumoto et al. 2001, Shao et al. 2002), environments in which ROS and NO are formed could be important determinants for triggering of bystander signals.

It has been suggested that the formation of bystander signals in irradiated cells does not emanate from the nucleus but from other organelles. Microbeam studies have clearly shown that the signals could be coming from extranuclear sources such as mitochondria or cell membranes (Shao et al. 2004, Tartier et al. 2007). ROS from the mitochondria are

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candidate triggers of bystander signaling, because cytoplasmic irradiation by an alpha-particle microbeam caused gene mutations that were repressed by DMSO treatment (Wu et al. 1999). The cell membrane is also a candidate site of bystander signal formation because filipin, an inhibitor of membrane signaling, suppressed the bystander effect by reducing the level of NO (Nagasawa et al. 2002, Shao et al. 2004).

Here, we tried to determine which types of radical species are involved in bystander signal formation. Nitric oxide and reactive oxygen species are thought to be scavenged by c-PTIO and DMSO, respectively (Matsumoto et al. 2001, Shao et al. 2004, Kashino et al. 2007a), and long-lived radicals that are the special radicals induced gradually after irradiation, are scavenged by ascorbic acid (Koyama et al. 1998). To this end, we used three radical scavengers, c-PTIO, DMSO and APM, using a method by which irradiated cells and bystander cells were independently treated with each radical scavenger. The results suggested that bystander effects were suppressed by the treatment of irradiated cells with c-PTIO; treatment of bystander cells with APM was also effective for suppression of bystander responses.

Materials and methods

Cell culture

Normal human diploid HE49 cells (passage 10–12) were cultured in Minimum Essential Medium (MEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories Inc., Logan, UT, USA) as described previously (Suzuki et al. 1998). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. Cells were treated with c-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imida-zoline-1-oxyl-3-oxide, Sigma-Aldrich, Tokyo, Japan), DMSO (dimethyl sulfoxide, Sigma-Aldrich), or APM (ascorbic acid phosphoric ester magnesium salt, Wako, Osaka, Japan). DMSO and distilled water were used as solvents for c-PTIO and APM, respectively. Cells were irradiated with X-rays from an X-ray generator (Softex, Tokyo, Japan) at 150 kVp and 5 mA with a 0.1-mm copper filter. The dose rates were 0.11 Gy/min for 0.01–0.1 Gy, and 0.49 Gy/min for 0.2–1.0 Gy, respectively.

Co-culture method

Cells (2×10^4) were seeded onto a 60×24 mm cover slip (Matsunami, Osaka, Japan) in a 100 mm dish one day before X-irradiation. Immediately after 1 Gy irradiation, cover slips containing irradiated cells were transferred to another 100 mm dish that was prepared under the same culture conditions; that is, irradiated cells on cover slips were co-cultured with non-irradiated cells on cover slips in a 100 mm dish. Irradiated cells were treated with the radical scavenger for 1 h before irradiation, and the reagent was present during irradiation. Immediately after irradiation, cells were washed twice with Phosphate-Buffered Saline (PBS) to remove the radical scavenger and transferred to the co-culture dish. In the case of treatment of the bystander cells with the radical scavenger, the latter was added at the time co-culturing began and was present throughout the co-culture period. After 24 h co-culture, only non-irradiated cells were used for the preparation of the micronuclei samples.

Micronucleus assay

After co-culture, the cover slip of irradiated cells was removed from the dish and non-irradiated bystander cells were analyzed in a micronucleus assay. The bystander cells were treated with 4 ml of hypotonic (0.1 M) potassium chloride (KCl, Wako) for 30 min, and fixed with 4 ml of methanol (Wako)-acetic acid (Wako) (5:1). The cells were incubated for 5 min, the supernatant was removed, and the cells were re-treated with 8 ml methanol-acetic acid solution and incubated on ice for 10 min. After treatment by a fixing solution, the cover slip was taken out from the dish, dried completely, and stained with 7.5% Giemsa (Merck Japan, Tokyo, Japan) for 40 min. Micronuclei per 2000 interphase cells were counted in each group.

Statistical analysis

The statistical analysis was performed using Student's *t*-test.

Results

Figure 1 shows a schema in our co-culture experiment. First, we examined how long co-culture is needed for sufficient induction of bystander effects in our protocol. The results showed that 6 h co-culture was sufficient time for the induction of bystander effects, and no increase in effects was observed by further incubation at least up to 48 h (Figure 2). Next, the X-ray dose dependency for induction of bystander effects was examined as shown in Figure 3. Saturation of the effect was observed above 0.1 Gy; therefore, it was thought that 1 Gy of X-irradiation would be sufficient for further study. We decided upon a protocol of 24 h co-culture with 1 Gy-irradiated

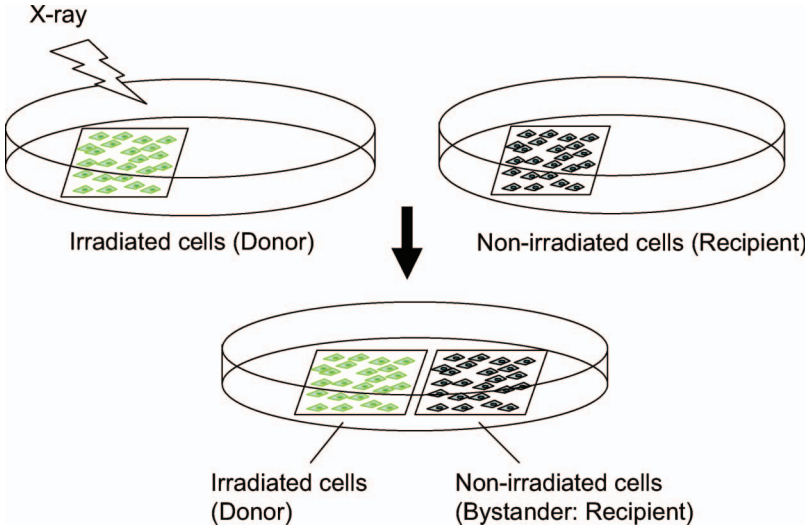

Figure 1. A schema of the co-culture experiment. Normal, non-irradiated human fibroblast cells were co-cultured with X-irradiated cells.

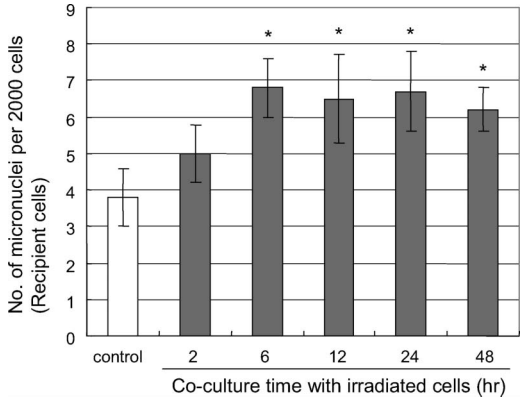

Figure 2. Micronuclei induction in non-irradiated cells co-cultured with 1 Gy-irradiated cells. Cells were co-cultured for the indicated time, and micronuclei samples were prepared from non-irradiated cells. Control means that cells were co-cultured with non-irradiated cells for 24 h. Result shows mean frequency ± standard error of the mean (SEM) of micronuclei in 1000 cells from three independent experiments. Significant differences were observed between the yields in control (co-cultured with non-irradiated cells) and those in 6- to 48-h co-culture periods (*Student's *t*-test $p < 0.05$).

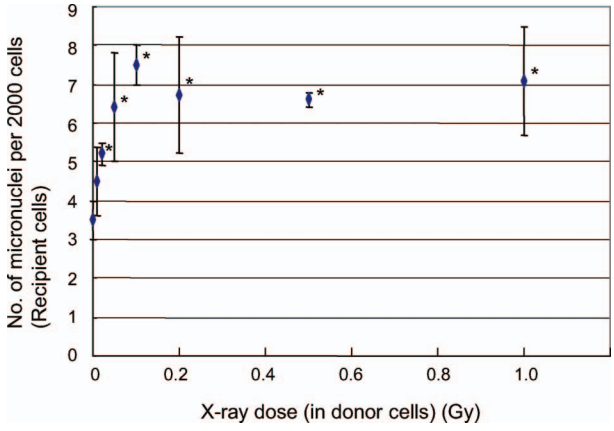

Figure 3. Micronuclei induction in non-irradiated cells co-cultured with cells irradiated at various doses. Cells were co-cultured for 6 h and micronuclei sample were prepared from non-irradiated cells. Result shows mean frequency ± standard error of the mean (SEM) of micronuclei in 1000 cells from three independent experiments. Significant differences were observed between the yield in controls (cells co-cultured with non-irradiated cells) and those in cells co-cultured with 0.01–1 Gy-irradiated cells (*Student's *t*-test $p < 0.05$).

cells to examine the suppressive effect of each radical scavenger on bystander effects.

We next examined the suppressive effects of radical scavengers on irradiated cells. Radical scavengers were added 1 h before 1 Gy irradiation and were present during the irradiation. When irradiated cells were treated with DMSO or APM, suppression of bystander effects were not observed (Figure 4a, 4b). On the other hand, when irradiated cells were treated with c-PTIO, known as an NO scavenger, for 1 h before 1 Gy irradiation, at concentrations between 5 to 50 μM c-PTIO treatments (Figure 4c). These results suggest that NO is related in an early step of bystander response.

Treatments of radical scavengers on bystander cells were also examined. Three radical scavengers were added after 1 Gy irradiation, that is, at the time the co-culturing began. These scavengers were present throughout the 24 h co-culture period; therefore, both irradiated cells (donors of bystander factors) and bystander cells (recipients of bystander factors) were treated with radical scavengers. In this

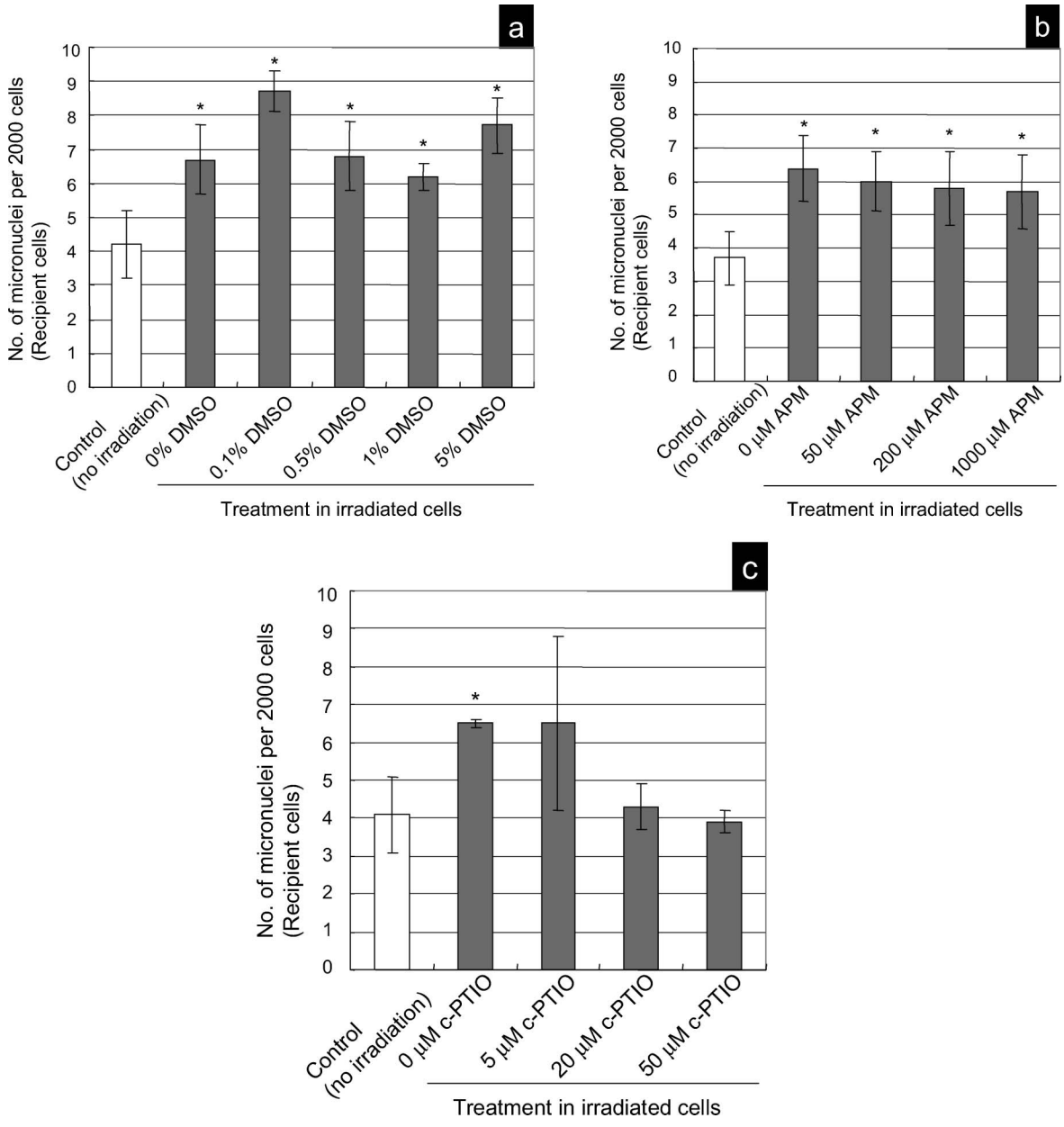

Figure 4. Micronuclei induction in non-irradiated cells co-cultured with 1 Gy-irradiated cells for 24 h. Irradiated cells for co-culture were treated with (a) DMSO, (b) APM, (c) c-PTIO for 1 h before X-irradiation. Results show mean frequency ± SEM of micronuclei in 1000 cells from three independent experiments. Significant differences were observed between the yield in control (cells co-cultured with non-irradiated cells) and those in indicated groups (*Student's *t*-test $p < 0.05$).

case, APM (1 mM) was effective at suppressing bystander micronuclei induction. It was suggested that ascorbic acid could suppress micronuclei induction in bystander cells where the signals had their effects. On the other hand, DMSO was not effective, and c-PTIO was only partially effective (Figure 5). In the case of c-PTIO, the suppressive effect was caused by the treatment of irradiated cells with c-PTIO. Therefore, we concluded that specific radical species that ascorbic acid can scavenge are involved in the micronuclei formation. Moreover, the NO scavenger c-PTIO was effective for suppression of the bystander effect only when it was used to treat irradiated cells.

Discussion and conclusion

In the present study, we examined which radical scavengers are effective for the suppression of bystander effects, and found that the NO scavenger c-PTIO was effective in the irradiated cells where the bystander signals are formed, whereas ascorbic acid is effective in the signal-receiving bystander cells. These results suggest that nitric oxide induced by

X-irradiation is involved in early process of bystander responses in irradiated cells, and hydroxyl radicals or long-lived radicals induced during bystander signal responses in bystander cells are the cause of micronuclei inductions. It is interesting that different types of radicals are involved in different parts of the bystander effect. The involvement of NO in bystander responses has been already reported by Matsumoto et al., and other groups (Matsumoto et al. 2001, Shao et al. 2002, 2008). It has been also reported that the increased levels of ROS can induce DNA damage in bystander cells (Yang et al. 2005). Our finding is strongly supported by previous reports and also suggests a new theory shown in Figure 6.

It is important to know which radicals are involved in the secretion of bystander factors. As DNA repair ability and the level of remaining DNA damage after X-irradiation are independent of this secretion (Kashino et al. 2007b), the secretion of bystander factors is thought to emanate by an extranuclear trigger. Following our results, NO induced by X-irradiation is a candidate for the secretion, because NO may be involved particularly in irradiated cells for suppression of bystander effects (Figure 4). We detected a high percentage of irradiated cells increasing their NO levels (data not shown). Shao et al. also demonstrated that increased NO is involved in bystander effects of fibroblasts (Shao et al. 2008), and they suggested that Transforming growth factor (TGF-β) is the secreted factor induced by NO production (Shao et al. 2008). In our experiment, it was not shown which factors were secreted as bystander factors. In our previous study, DMSO treatment of irradiated cells effectively suppressed the secretion of the unknown bystander factor (Kashino et al. 2007a). Therefore, radical reactions related in NO and ROS in irradiated cells may depend on the secretion of cytokines as a bystander factor. However, it is not clear why DMSO was not effective in this study, while bystander effects were completely suppressed by DMSO in CHO cells (Kashino et al. 2007a). It was reported that DMSO was not effective for the suppression of bystander effects induced by alpha particle microbeam irradiation (Zhou et al. 2000). One of the reasons for this disagreement may be that the actions of DMSO are different in the types of cells and the conditions of irradiated cells. Vines et al. suggested that glucose 6-phosphate dehydrogenase (G6DP) is involved in the production of bystander signals (Vines et al. 2008). This suggests that reactive oxygen species may be not directly but indirectly involved in the production of bystander signal.

It is known that APM is a suitable for continuous treatment of ascorbic acid on cells, because an APM changes to an ascorbic acid by esterase in the cells or culture medium (Kashino et al. 2003). Following the results in the present study, we understood that ascorbic acid is a suitable radical scavenger for suppression of the bystander response in signal-receiving bystander cells. It is interesting that ascorbic acid was effective while DMSO was not. This suggests that hydroxyl radicals, scavenged by DMSO, are not involved in bystander cells. Our group has been studying long-lived radicals that are scavenged by ascorbic acid (Koyama et al. 1998, Kumagai et al. 2003); we (Koyama et al. 1998) and others (Waldren et al. 2004) have found that gene mutation and transformation induced by X-irradiation are suppressed by ascorbic acid treatment. Also, we reported that ascorbic acid is effective for suppression of cellular senescence of normal human fibroblasts (Kashino et al. 2003). These cellular events in normal aging and radiation response are thought to be strongly related in homeostasis

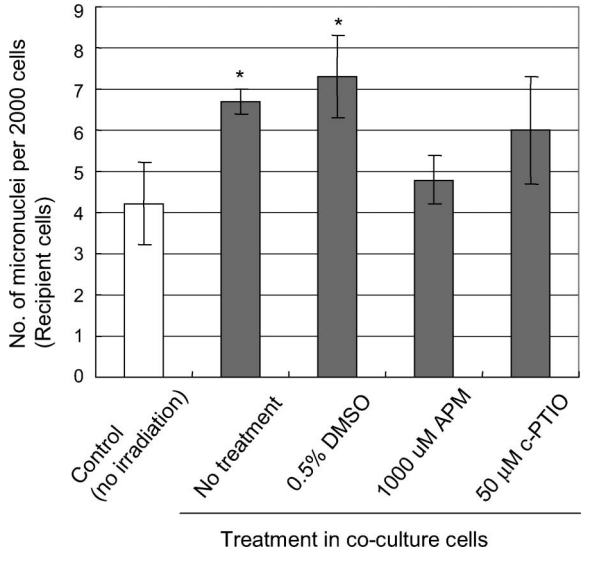

Figure 5. Micronuclei induction in non-irradiated cells co-cultured with 1 Gy-irradiated cells for 24 h. Irradiated cells were co-cultured with non-irradiated HE49 cells in medium containing 0.5% DMSO, 1000 μM APM or 50 μM c-PTIO for 24 h. Result shows mean frequency $\pm$ SEM of micronuclei in 1000 cells from three independent experiments. Significant differences were observed between the yield in control (cells co-cultured with non-irradiated cells) and those in indicated groups (*Student's t-test $p < 0.05$).

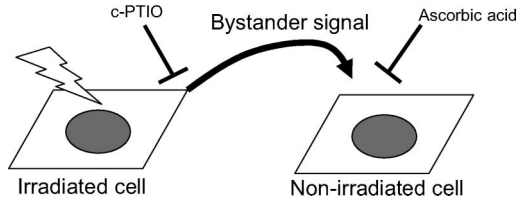

Figure 6. Summary of the present study. Treatment on X-irradiated cells with c-PTIO is effective for the suppression of the entire bystander response. On the other hand, ascorbic acid treatment is effective for suppression of bystander signal-induced micronuclei only in signal recipient cells.

mechanisms based on cell-to-cell communication. It is possible that these effects of ascorbic acid enhance cellular communication and homeostasis, although further studies are needed to know the involvements of radical species on the responses in bystander cells. In additional analysis of bystander effects, it is important to remark the exact properties of cultured human fibroblasts and the real effects of ascorbic acid.

In conclusion, radiation induced bystander effects are related in inductions of radical species but the roles of the radical species are different between irradiated cells and bystander 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.

References

Hamada N, Matsumoto H, Hara T, Kobayashi Y. 2007. Intercellular and intracellular signaling pathways mediating ionizing radiation-induced bystander effects. Journal of Radiation Research 48:87–95.

Iyer R, Lehnert BE, Svensson R. 2000. Factors underlying the cell growth-related bystander responses to alpha particles. Cancer Research 60:1290–1298.

Kashino G, Kodama S, Nakayama Y, Suzuki K, Fukase K, Goto M, Watanabe M. 2003. Relief of oxidative stress by ascorbic acid delays cellular senescence of normal human and Werner syndrome fibroblast cells. Free Radical Biology and Medicine 35:438–443.

Kashino G, Prise KM, Schettino G, Folkard M, Vojnovic B, Michael BD, Suzuki K, Kodama S, Watanabe M. 2004. Evidence for induction of DNA double strand breaks in the bystander response to targeted soft X-rays in CHO cells. Mutation Research 556:209–215.

Kashino G, Prise KM, Suzuki K, Matsuda N, Kodama S, Suzuki M, Nagata K, Kinashi Y, Masunaga S, Ono K, Watanabe M. 2007a. Effective suppression of bystander effects by DMSO treatment of irradiated CHO cells. Journal of Radiation Research 48:327–333.

Kashino G, Suzuki K, Matsuda N, Kodama S, Ono K, Watanabe M, Prise KM. 2007b. Radiation induced bystander signals are independent of DNA damage and DNA repair capacity of the irradiated cells. Mutation Research 619:134–138.

Koyama S, Kodama S, Suzuki K, Matsumoto T, Miyazaki T, Watanabe M. 1998. Radiation-induced long-lived radicals which cause mutation and transformation. Mutation Research 421:45–54.

Kumagai J, Masui K, Itagaki Y, Shiotani M, Kodama S, Watanabe M, Miyazaki T. 2003. Long-lived mutagenic radicals induced in mammalian cells by ionizing radiation are mainly localized to proteins. Radiation Research 160:95–102.

Matsumoto H, Hayashi S, Hatashita M, Ohnishi K, Shioura H, Ohtsubo T, Kitai R, Ohnishi T, Kano E. 2001. Induction of radioresistance by a nitric oxide-mediated bystander effect. Radiation Research 155:387–396.

Mothersill C, Seymour C. 1997. Survival of human epithelial cells irradiated with cobalt 60 as microcolonies or single cells. International Journal of Radiation Biology 72:597–606.

Nagasawa H, Little JB. 1992. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Research 52:6394–6396.

Nagasawa H, Cremesti A, Kolesnick R, Fuks Z, Little JB. 2002. Involvement of membrane signaling in the bystander effect in irradiated cells. Cancer Research 62:2531–2534.

Prise KM, Belyakov OV, Folkard M, Michael BD. 1998. Studies of bystander effects in human fibroblasts using a charged particle microbeam. International Journal of Radiation Biology 74:793–798.

Sawant SG, Randers-Pehrson G, Geard CR, Brenner DJ, Hall EJ. 2001. The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells *in vitro* can be initiated in the unirradiated neighbors of irradiated cells. Radiation Research 155:397–401.

Shao C, Furusawa Y, Aoki M, Matsumoto H, Ando K. 2002. Nitric oxide-mediated bystander effect induced by heavy-ions in human salivary gland tumour cells. International Journal of Radiation Biology 78:837–844.

Shao C, Folkard M, Michael BD, Prise KM. 2004. Targeted cytoplasmic irradiation induces bystander responses. Proceedings of the National Academy of Sciences of the USA 101:13495–13500.

Shao C, Folkard M, Prise KM. 2008. Role of TGF-beta1 and nitric oxide in the bystander response of irradiated glioma cells. Oncogene 27:434–440.

Suzuki K, Takahara R, Kodama S, Watanabe M. 1998. *In situ* detection of chromosome bridge formation and delayed reproductive death in normal human embryonic cells surviving X irradiation. Radiation Research 150:375–381.

Tartier L, Gilchrist S, Burdak-Rothkamm S, Folkard M, Prise KM. 2007. Cytoplasmic irradiation induces mitochondrial-dependent 53BP1 protein relocalization in irradiated and bystander cells. Cancer Research 67:5872–5879.

Vines AM, Lyng FM, McClean B, Seymour C, Mothersill CE. 2008. Bystander signal production and response are independent processes which are cell line dependent. International Journal of Radiation Biology 84:83–90.

Waldren CA, Vannais DB, Ueno AM. 2004. A role for long-lived radicals (LLR) in radiation-induced mutation and persistent chromosomal instability: Counteraction by ascorbate and RibCys but not DMSO. Mutation Research 551:255–265.

Wu LJ, Randers-Pehrson G, Xu A, Waldren CA, Geard CR, Yu Z, Hei TK. 1999. Targeted cytoplasmic irradiation with alpha particles induces mutations in mammalian cells. Proceedings of the National Academy of Sciences of the USA 96:4959–4964.

Yang Z, Asaad N, Held KD. 2005. Medium-mediated intercellular communication is involved in bystander responses of X-ray-irradiated normal human fibroblasts. Oncogene 24: 2096–2103.

Zhou H, Randers-Pehrson G, Waldren CA, Vannais D, Hall EJ, Hei TK. 2000. Induction of a bystander mutagenic effect of alpha particles in mammalian cells. Proceedings of the National Academy of Sciences of the USA 97:2099–2104.