Effective Suppression of Bystander Effects by DMSO Treatment of Irradiated CHO Cells

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INTRODUCTION

It has recently been shown that non-irradiated cells are affected by the signals from irradiated cells.1–5 This bystander effect is thought to be important for risk estimation of radiation carcinogenesis as it predominates at low dose.6 Many reports concerning the bystander effects have been published in recent years,7–15 but the mechanisms are not fully understood. It is important to determine what is the trigger for the formation of the bystander response, because it is unclear what is involved in the initiation process. As some papers suggest that reactive oxygen species (ROS) and nitric oxide (NO) are related in the bystander response,9–14 the environment in which ROS and NO are formed could be an important determinant for triggering of bystander signals.

It is suggested that the formation of bystander signals in irradiated cells does not emanate from the nucleus but from other organelles.14 Microbeam studies have clearly shown that the signals could be coming from extranuclear sources such as mitochondria or cell membrane. ROS from mitochondria is a candidate for the trigger of a bystander signal, because cytoplasmic irradiation by alpha particle microbeam caused gene mutations that are repressed by DMSO treatment.15 The cell membrane is also a candidate for the trigger of bystander signal formation, because filipin, an inhibitor of ceramide dependent signals suppressed the bystander effects through the reduced level of NO.14,16

DMSO is well known as a radical scavenger.17–20 Researchers have used DMSO as a radioprotective regent. DeLara et al reported that DMSO suppresses the induction of...
DNA double strand breaks by ionizing radiation, and it was suggested that initial yields of double strand breaks were decreased by the presence of DMSO during irradiation.\textsuperscript{19} Our group also reported that lethal effects by ionizing radiation such as cells death and chromosome aberration were suppressed by DMSO treatment before irradiation.\textsuperscript{18,20} These effects are thought to be due to scavenging of short-lived active radicals such as OH and H radicals that are thought to act on DNA. In addition, it has been reported that DMSO is able to induce differentiation in leukemia cells and in lung carcinoma cells,\textsuperscript{21-23} suggesting that signal formations from membrane are changing. In the radiation biology field, the actions of DMSO on extranuclear organelles such as cell membrane have not been fully characterized in cultured cells.

The main purpose in our study is to understand the mechanisms of the radiation induced bystander response in irradiated cells in CHO cells. In order to evaluate bystander responses with a more sensitive method, we used repair deficient xrs5 cells as shown in our previous report.\textsuperscript{5} In the present study, we examined whether DMSO treatment is effective for the suppression of bystander signal formation. The main approach in this study is that only irradiated cells but not bystander cells are treated with low concentrations of DMSO, and we found that the treatment provides an efficient suppression of inductions of bystander effects. Because the observations of ‘new targets’ of ionizing radiation for induction of micronuclei has brought about a paradigm shift of classical radiobiological theory, we should also reconsider the actions of radical scavengers such as DMSO on irradiated cell populations.

MATERIALS AND METHODS

Cell culture
Chinese hamster ovary (CHO) cells and xrs5 cells were kindly supplied by Dr Tom K. Hei, Columbia University, New York. Cells were cultured in MEM alpha medium (Invitrogen Ltd, California) supplemented with 10% FBS (HyClone Laboratories, Inc., Logan, UT, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen Ltd, California, USA). Cells were maintained at 37°C in a humidified atmosphere with 5% CO\textsubscript{2}.

X-irradiation and micronucleus assay
To investigate the induction of micronuclei by direct X-irradiation, the cells were irradiated with conventional X-rays. Exponentially growing cells in 6-well plates were irradiated with X-rays using an X-ray generator (Pantak IV at Gray Cancer Institute) 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. Immediately after irradiation, cells were treated with 2 μg/ml cytochalasin B for 24 h. When cells were treated with DMSO for 1 hr before and during irradiation, DMSO was removed at the same time as adding cytochalasin B. 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 20 min. Micronuclei per 1,000 binucleated cells were counted.

Medium transfer experiment
Cells (CHO or xrs5 ; 5 × 10\textsuperscript{4}) were seeded onto 6 well plates one day before X-irradiation. Fifty minutes before irradiation medium was changed to DMSO containing medium and incubated. As it took about 10 min for entire X-irradiation and DMSO were kept in the medium during irradiation, cells were treated with DMSO for 1 hr. Cells were irradiated with 1 Gy of conventional X-rays. Immediately after irradiation, medium was changed to normal medium and cells were washed with medium in order to remove DMSO from the medium. Then cells were incubated for 24 hrs following irradiation in order to prepare the conditioned medium. After the incubation, the conditioned medium was filtered through a 0.22 μm filter and transferred to unirradiated cultured cells on 6 well plates that were incubated for 2 days at the time. 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. 2,000 binucleated cells were observed in each sample.

Co-culture experiment
xrs5 cells (5 × 10\textsuperscript{4}) were seeded onto a 22 × 22 mm cover slip in a 60 mm one day before X-irradiation. Immediately after 1 Gy - irradiation, a coverslip containing irradiated cells was transferred to another 60 mm dish that was prepared under the same culture condition, that is, irradiated cells on coverslip were co-cultured with non-irradiated cells on coverslip in the same 60 mm dish. When irradiated cells were treated with DMSO for 1 hr before and during irradiation, DMSO was removed just before coculture. 24 hour’s co-culture was done in the medium containing cytochalasin B, and a coverslip containing irradiated cells was removed. Then only non-irradiated cells were used for the preparation of the micronuclei samples. Micronucleus samples were prepared as described above. 2,000 binucleated cells were observed in each sample.

DCFH Assay
Cells were treated with 1 μM DCFH-DA (Molecular Probes) in PBS solution for 30 min. Then, cells were suspended in PBS solution and the fluorescent intensity of the 2', 7' - dichlorofluorescin was measured with a fluorescence...
spectrophotometer F2000 (Hitachi, Tokyo, Japan). The excitation and emission wavelengths used were 503 nm and 524 nm, respectively. Cells in suspension in a tube, after DCFH-DA treatment, were irradiated with X-rays and fluorescent intensities were measured immediately after irradiation. When cells were treated with DMSO, cells were treated 0.5% DMSO in normal medium for 30 min and then medium were changed to PBS solution containing DCFH and DMSO for further incubation for 30 min. In the assay with FACscan (Becton Dickinson), cells in T25 were treated with 5 μM DCFH solution in PBS for 30 min and cells were harvested to pour into the scan.

**Statistical analysis**

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

**Fig. 1.** Effect of DMSO treatment on induced micronuclei in 1 Gy-irradiated CHO (a) and 0.2 Gy-irradiated xrs5 cells (b). Cells were treated with 0.5% or 1.0% DMSO for 1 hr and DMSO was present during X-irradiation. Results in X-irradiated condition show mean numbers of micronuclei ± SEM, per 1000 binucleated cells from three independent experiments. A significant difference was observed between non-treated CHO and DMSO treated CHO after X-irradiation (Student’s *t* test *p* < 0.05).

**Fig. 2.** Micronuclei induction in xrs5 cells exposed to conditioned medium from irradiated CHO cells (a) and xrs5 cells (b), and the effect of DMSO. Cells were treated with 0.5% DMSO for 1 hr and DMSO was present during X-irradiation. Results show mean numbers of micronuclei ± SEM, per 2000 binucleated cells from three independent experiments. Significant differences were observed between non-irradiated control cells and irradiated control cells in two cell lines (* Student’s *t* test, *p* < 0.05). Significant differences were also observed between irradiated control cells and irradiated 0.5% DMSO treated cells in xrs5 cells (** Student’s *t* test, *p* < 0.05).
RESULTS

As shown in Fig. 1, micronuclei inductions were observed after 0.2 and 1.0 Gy irradiation in CHO and xrs5 cells, respectively. These doses were determined as inducing similar levels of micronuclei in each cell line. Interestingly, the suppressive effects by 0.5% or 1% DMSO were different between CHO cells and xrs5 cells. In the case of CHO, about 20% of micronuclei induction was suppressed by 0.5% or 1% DMSO treatment, whereas no suppressive effects were observed in xrs5 cells. When xrs5 cells were irradiated with 0.5 or 1 Gy, no suppressive effects by 0.5% DMSO treatment were also observed (data not shown). We checked for morphological changes after DMSO treatment for 1 hour, and we observed that a concentration of 3% caused morphological changes. Therefore, we used < 1% concentration of DMSO for 1 hr treatment.

As shown Fig. 1, we examined the effects of DMSO on the induction of micronuclei in irradiated cells. No significant differences in its effects were observed between 0.5% and 1.0% concentration of DMSO in CHO. Therefore, we
used only 0.5% concentration in the experiments for bystander effects. Bystander effects were estimated using medium transfer and co-culture methods. As shown in Fig. 2 and 3, micronuclei inductions were observed in non-irradiated bystander cells. Bystander effects shown as induction of micronuclei in non-irradiated xrs5 cells were similar in both cases between irradiation of CHO and irradiation of xrs5 in medium transfer method (Fig. 2a and 2b). Also, induced levels of micronuclei in bystander xrs5 cells were similar between the medium transfer method and the co-culture method (Fig. 2b and 3). These results suggest that both methods we used were suitable for an estimation of suppressive effects of DMSO on typical bystander effects. The results showed that the complete suppression of the inductions of micronuclei was observed in bystander cells by the treatment of DMSO on irradiated cells (Fig. 2 and 3). These results suggest that DMSO can suppress the formation of bystander signal during the early stage of bystander responses. We thought that this suppressive effect of DMSO on bystander effects is correlated with removal of reactive oxygen species (ROS) induced by X-irradiation. Therefore, we examined oxidative stress levels immediately after irradiation using the DCFH assay. As shown in Fig. 4, oxidative stress levels immediately after X-irradiation were increased in a dose dependent manner in CHO cells and xrs5 cells. Next, we examined the suppressive effect of DMSO on oxidative stress in irradiated cells. Fig. 5 and Fig. 6 showed that 0.5% DMSO treatment for 1 hr is not adequate for suppressing oxidative stress in CHO cells. However, the suppression of DCF value was shown when cells were treated with higher DMSO (2%), or 1 mM ascorbic acid phosphorylated ester (APM), 1 mM ascorbic acid 2-glucoside (AA-2G) (Fig. 6). As shown in Fig. 2 and 3, bystander effects were completely suppressed by the treatment of 0.5% DMSO, therefore it is concluded that there is no correlation between bystander signal formation and intracellular oxidative stress after X-irradiation in irradiated cells.

**DISCUSSIONS**

Shao et al. reported that cytoplasmic irradiation by a microbeam causes micronuclei in the co-cultured non-irradiated cells, and the levels of intercellular bystander effects are similar between cytoplasmic irradiation and nuclear irradiation. Also, we found that the levels of bystander signal from irradiated cells are not different between CHO and xrs5 cells, who have different repair capacity for DNA double strand breaks.25) Therefore, it is suggested that levels of bystander signals from irradiated cells are not coming from DNA double strand breaks, and are independent of the number of remaining DNA double strand breaks induced by ionizing radiation.

In the present study, we can conclude that there is no correlation between ROS and bystander signal formation in irradiated cells, because 0.5% DMSO treatment was not sufficient to suppress oxidative stress induced by ionizing radiation, in spite of its effective suppression of bystander effects. Therefore, it is suggested that transient radical formation immediately after irradiation is not the origin of the bystander signal. It is interesting that the suppressive effects of DMSO treatment on micronuclei induction in irradiated cells are different between CHO and xrs5 (Fig. 1). One possible mechanism for this phenomenon is that DMSO treatment enhances repair activity of the non-homologous end...
joining pathway. As ku80 is defective in xrs5, it is impossible to enhance repair activity by DMSO treatment. On the other hand, higher concentrated DMSO (5%) could suppress the induction of micronuclei in irradiated xrs5 (data not shown). Therefore, it is thought that there are two independent radioprotective actions of DMSO, that is, at lower concentrations DMSO acts as activating factor for a radioprotective signal, while at higher concentration DMSO acts as radical scavenger. As bystander effects were affected by the lower concentration of DMSO (0.5%), it is suggested that effects of DMSO against signal activation for bystander response in irradiated cells are correlated with activation of radioprotective signal by DMSO in irradiated cells. Secreting cytokines and the ceramide dependent pathway are candidates for the radioprotective response through cell-cell communication via the culture medium as suggested by Shao et al. 14)

In the DCFH assay, we could determine the oxidative stress levels indicating the intracellular amounts of reactive radical species immediately after irradiation in a dose dependent manner (Fig. 4). The linear relationship between X-ray dose and DCFH fluorescent values suggests that the energy deposition through photon tracks give radical species without saturation up to 1 Gy (Fig. 4). On the other hand, it is reported that bystander effects are saturated at lower doses (0.2–0.3 Gy). 2627 Therefore the capacity of the DCFH assay to determine oxidative stress is sensitive enough for 1 Gy-irradiation which we used in the study for bystander effects. Although 0.5% DMSO treated cells showed similar oxidative levels to non-treated cells in unirradiated and irradiated conditions (Fig. 5), oxidative levels were suppressed in ascorbic acid (APM and AA-2G) treated cells in irradiated condition (Fig. 6). Therefore this approach is sensitive enough to determine the suppressive effects of DMSO on oxidative levels. However, we cannot exclude the possibility that small undetectable changes of oxidative status by DCFH method are involved in signaling of the bystander response. Further study is needed to determine which specific extranuclear organelles, such as mitochondria and cellular membranes, are involved in triggering the bystander signal.

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