



# Influence of catechins on bystander responses in CHO cells induced by alpha-particle irradiation

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## ABSTRACT

In this work, we studied alpha-particle induced and medium-mediated bystander effects in Chinese hamster ovary (CHO) cells through micronucleus (MN) assay. We showed that signal transduction from irradiated cells to bystander cells occur within a short time after irradiation. We then studied the effects of ROS (reactive oxygen species)-scavenging catechins in the medium before irradiation. We observed decreases in the percentage of bystander cells with MN formation and thus proved the protection effect of catechins on bystander cells from radiation.

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## 1. Introduction

Ionizing radiation has been shown to initiate the biological production of reactive oxygen species (ROS), including oxygen ions, free radicals and peroxides, in human cells. Ionizing radiation induces cell death, chromosome aberration and gene mutation (Coates et al., 2004) and it is widely accepted that most of these biological effects are caused by free radicals such as hydroxyl and hydrogen radicals (Chaudhry, 2006).

Catechins, a polyphenolic compound of green tea, are well known as scavengers of ROS in human or mammalian cells (Yang et al., 1994; Rice-Evans et al., 1996; Valcic et al., 2000). (-)-epigallocatechin gallate (EGCG), which is a major constituent of green tea catechins, is the most active antioxidant component with its effects seen at micromolar concentrations (Anderson et al., 2001). The biological benefits of EGCG are generally attributed to their antioxidant activity to scavenge free-radical oxygen (Rice-Evans et al., 1996; Valcic et al., 2000).

Radiation-induced bystander effects refer to the induction of biological effects in cells that are not directly traversed by an ionizing radiation. Earlier studies showed that bystander effects could be induced by treatment of unirradiated cells with medium taken from cell cultures previously exposed to irradiation (Mothersill and Seymour, 1997, 1998; Lyng et al., 2002). Although the underlying mechanisms of the bystander effect are not well understood, some evidence suggests that ROS is involved in different systems (Azzam et al., 2001, 2002).

Many researchers have reported the effect of EGCG on scavenging ROS in irradiated cells with low linear energy transfer

(LET) radiation such as X-rays (Yang et al., 1994; Anderson et al., 2001). However, to the best of our knowledge, no investigations have been made on the effect of EGCG on scavenging ROS in bystander cells induced by a high LET radiation such as alpha particles. In the present study, the Chinese hamster ovary (CHO) cells will be irradiated with alpha particles and the culture medium will be transferred to the bystander cells. The effect of EGCG in bystander cells on reducing micronucleus (MN) formation will be investigated.

## 2. Materials and methods

### 2.1. Cell culture

The Chinese hamster ovary (CHO) cells were kindly provided by Tom K. Hei, Columbia University, New York. The cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> with MEM (Gibco): F-12 (Gibco) (1:1) mixed medium supplemented with 8% FCS (Gibco), 2 × 10<sup>-4</sup> M Glycine (Sigma), 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco).

### 2.2. Alpha-particle irradiation

Mylar-film based cell-culture Petri dishes were used in the medium transfer experiments. Mylar films with a thickness of 3.5 μm were glued with epoxy (Araldite<sup>®</sup> Rapid, England) to the bottom of Petri dishes (Orange Scientific) with a diameter of 5 cm, with a hole of 1 cm diameter drilled at the bottom. The alpha-particle source employed was a planar <sup>241</sup>Am source (with an activity of 1.85 × 10<sup>5</sup> Bq (5 μCi)). The Mylar film based cell dishes were irradiated from the bottom at a dose rate of 0.3 cGy/s as shown in Fig. 1 (Chan et al., 2008).

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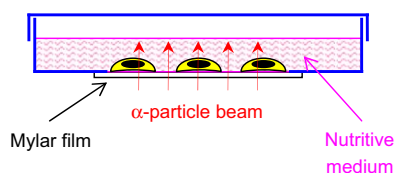


Fig. 1. The irradiation of the cell monolayer through the Mylar-film based cell dish.

### 2.3. Medium transfer experiment

For the study of the effect of time on MN formation in bystander cells,  $2 \times 10^4$  cells in 5 ml of medium from a confluent culture were seeded on the Mylar film to be irradiated cells, and  $1.2 \times 10^4$  cells in 2 ml of medium were seeded in  $\varnothing 35$  mm culture dish to be bystander cells. After irradiation, the irradiated cells were returned to the  $37^\circ\text{C}$  incubator immediately. The culture medium was then filtered through a  $0.22\ \mu\text{m}$  filter and 2 ml of the medium was transferred to each unirradiated sample at different time after irradiation.

For the study of the effect of EGCG on MN formation in bystander cells, 3 h before irradiation, EGCG (Sigma) was added to the culture medium from the stock solution (10 mM) in DMSO to give the appropriate final concentrations. Immediately before irradiation, the medium was changed and the cells were irradiated for 60 s. At appropriate time after irradiation, the culture medium was transferred to the unirradiated cells and the irradiated cells were transferred into  $\varnothing 35$  mm culture dish. EGCG with different concentrations was added to the medium for both irradiated and bystander cells. The control samples were treated in the same way but were not irradiated.

### 2.4. Micronucleus assay

The frequency of MN formation was measured by the cytokinesis block technique (Fenech, 2000). For both experiments, after transferring the culture medium into bystander cells, Cytochalasin B (CB, Sigma) was added into the culture medium for both irradiated and bystander cells at the final concentration of  $2.5\ \mu\text{g}/\text{ml}$  at 4–6 h post cell seeding and the cultures were incubated at  $37^\circ\text{C}$  (Azzam et al., 2001, 2002). After 24 h, the cells were fixed with methanol:acetic acid (9:1 v/v). After air drying, the cells were stained with 0.1% acridine orange (Sigma) for 5 min, and viewed under a fluorescence microscope (Fenech, 2000). At least 500 binucleated cells in at least 10 fields of view were examined.

### 2.5. Detection of ROS

HDCFDA (2',7'-dichlorodihydrofluorescein diacetate)-loaded cells were seeded on the Mylar film which was glued to the bottom of a steel ring and placed in a 24-well plate. All the cells on the Mylar film were irradiated. The 24-well plate was then put into a microplate fluorescence reader with temperature maintained at  $37^\circ\text{C}$ . The excitation filter was set at 486 nm and the emission filter was set at 520 nm. The fluorescence from each well was captured, digitized, and stored on a computer using Fluostar Optima. Data points were taken every 2 min for 10 min and the data were exported to Microsoft<sup>®</sup> Excel<sup>®</sup> software for analysis.

### 2.6. Statistical analyses

Statistical analyses (e.g., performance of student's t-test, calculation of standard deviations) were carried out using the Microsoft<sup>®</sup> Excel<sup>®</sup> software.

## 3. Results

### 3.1. Time dependence of micronucleus formation in bystander cells

To determine the time dependence of MN formation in bystander cells, the culture medium holding the irradiated cells was transferred to bystander cells at different time after irradiation, and then the bystander cells were fixed for the MN assay for 24 h after the start of the medium transfer.

Fig. 2 shows the MN induction in bystander cells when the culture medium was transferred at different times after the cells were exposed to an alpha-particle radiation dose of 18 cGy. Compared to the corresponding unirradiated controls, when the culture medium was transferred immediately (0 h) from the irradiated cells after irradiation, the MN induction was slightly higher ( $P=0.0865$ ) than those for medium transfers at 0.5, 1 and 3 h after irradiation. The MN induction decreased if the waiting time before starting the medium transfer increased. This suggests that the signal transduction from irradiated cells to bystander cells occur within a short time after irradiation. These data have also been reported separately by Wong et al. (2009).

### 3.2. Effect of EGCG on bystander cells after alpha-particle irradiation

To investigate the effect of EGCG on MN formation in both irradiated and bystander cells, different concentrations of EGCG were added to the medium holding the cells to be irradiated 3 h before irradiation. Immediately after irradiation, the medium was transferred to the bystander cells and EGCG with different concentrations was added to the medium for both irradiated and bystander cells and then fixed for the MN assay for 24 h after the start of the medium transfer.

Fig. 3A shows the effect of EGCG on MN formation in irradiated cells. Compared to the irradiated samples without any treatment ( $0\ \mu\text{M}$ ), the decreases in MN induction were not significant for concentrations of 1, 10 and  $20\ \mu\text{M}$ . However, the effect of EGCG on the MN induction in bystander cells was somewhat different (Fig. 3B). While all bystander samples consistently showed decreased MN yields significantly compared to the bystander samples without any treatment ( $0\ \mu\text{M}$ ), the decreases appeared to be dependent on the concentration of EGCG. The MN induction in bystander cells was between 1.35- and 1.68-fold lower than

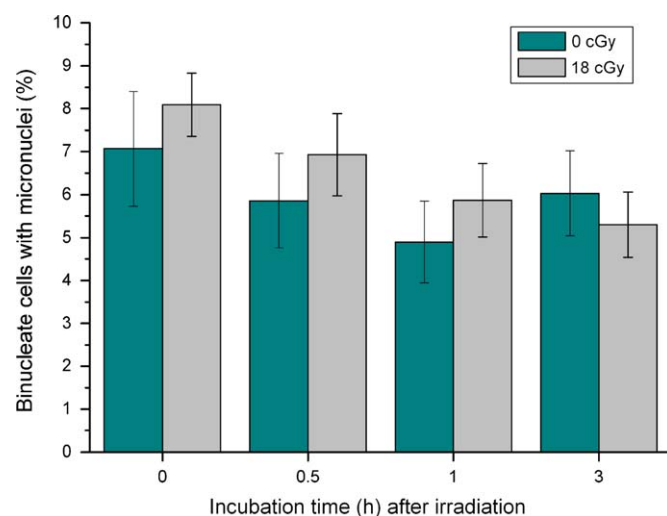


Fig. 2. Micronucleus induction in bystander CHO cells as a function of incubation time after irradiation. Results are means of three independent experiments  $\pm$  s.d. More micronucleus induction is found in bystander CHO cells than in the controls for the 0 and 1 h time points with statistical significance ( $p < 0.05$ ).

controls (0  $\mu\text{M}$ ), with significant decrease ( $P=5.4 \times 10^{-5}$ ) for the EGCG concentration of 20  $\mu\text{M}$ .

### 3.3. Reduction of ROS in irradiated cells treated with EGCG

For the medium transfer experiments, no gap junction communication exists between the irradiated and bystander cells. Hence soluble factors must be involved in the medium-mediated bystander effects. To examine the scavenging effect of EGCG on ROS in CHO cells induced by alpha-particle irradiation, different

concentrations of EGCG were added 3 h before irradiation. HDCFDA was used as a probe with microplate fluorescence reader to examine the generation of ROS in CHO cells by alpha-particle irradiation.

Table 1 shows the levels of ROS corresponding to the addition of different concentrations of EGCG in both irradiated and unirradiated samples. Compared to the corresponding control samples without any treatment (of which the ROS levels are set as 1), the reduction in ROS levels by EGCG is more effective in irradiated cells than in the unirradiated cells.

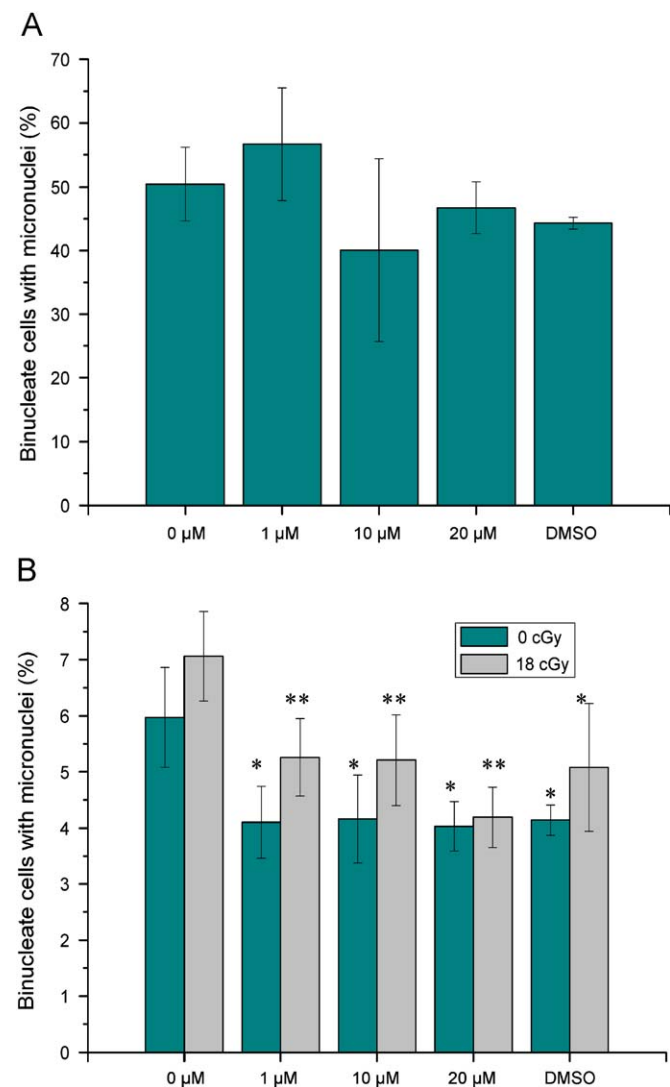
## 4. Discussion

In the present study, we have shown that the signal transduction from irradiated cells to bystander cells occurs within a short time after irradiation. This result suggests that the signaling molecules secreted by irradiated cells at early times after irradiation are crucial to the initiation of bystander responses (Yang et al., 2007). Once the time of maximal signal transduction is found, the effect of EGCG on bystander cells can be examined.

Fig. 3A shows that the decreases in MN induction in irradiated cells were not significant for all EGCG concentrations, while Fig. 3B shows significant decreases in MN induction in bystander cells with EGCG. These results suggest that DNA damages in directly irradiated cells are different from those induced in bystander cells (Huo et al., 2001). Micronuclei have been suggested to arise predominantly from non-rejoined DNA double strand breaks (DSBs) (Fimognari et al., 1997). In irradiated cells, the DNA damages are mainly caused by direct damages other than through ROS attack. However, in bystander cells, the ROS scavenger EGCG is found to be active in reducing the amount of MN induction by scavenging the ROS before they react with the DNA. This suggests that ROS are involved in the alpha-particle radiation-induced bystander effects.

ROS, such as superoxide, hydrogen peroxide and NO, are not effective at causing DNA DSBs (Olive and Johnston, 1997), thus the MN induction in bystander cells are likely the result from downstream reactions of  $\text{O}_2^-$ ,  $\text{H}_2\text{O}_2$  and  $\cdot\text{OH}$ . A previous study showed the scavenging effect of EGCG (20  $\mu\text{M}$ ) in  $\text{O}_2^-$  reached about 80% in the first 5 min (Yang et al., 1994). Some studies also found that EGCG reduced the amount of prompt DNA strand breaks, at the physiologically significant concentrations of 1–2  $\mu\text{M}$  (Anderson et al., 2001). In our study, the scavenging effect of EGCG in 20  $\mu\text{M}$  on bystander cells is the most significant when compared to EGCG concentrations of 1 and 10  $\mu\text{M}$ . The results suggest that scavenging of ROS induced by alpha-particle irradiation in the case of bystander CHO cells is more effective with higher concentrations of EGCG. It is remarked here that the kind of ROS induced and the production rate of ROS vary with different types of cells and radiations.

In this study, 0.04% of DMSO was added to the culture medium in both irradiated and bystander cells. Fig. 3B shows that in both irradiated and unirradiated samples, there are significant decreases in MN induction with the addition of DMSO. Since DMSO is also an antioxidant which has been found to inhibit the induction of mutations with alpha-particle irradiation (Wu et al., 1999), it is important to study whether the decrease in MN



**Fig. 3.** Micronucleus induction in directly irradiated and bystander CHO cells as a function of concentration of EGCG and DMSO after exposed to 18 cGy irradiation. Panel A: Micronucleus induction in directly irradiated CHO cells as a function of concentration of EGCG and DMSO. Panel B: Micronucleus induction in bystander CHO cells as a function of concentration of EGCG and DMSO. Results are means of three independent experiments  $\pm$  s.d. \* $P < 0.05$ , \*\* $P < 0.005$  compared to the corresponding control (without any treatment).

**Table 1**  
ROS levels (arbitrary units) in directly irradiated CHO cells with treatments of EGCG and DMSO.

Dose (cGy)	EGCG (0 $\mu\text{M}$ )	EGCG (1 $\mu\text{M}$ )	EGCG (10 $\mu\text{M}$ )	EGCG (20 $\mu\text{M}$ )	0.04% DMSO
0	1	0.94 $\pm$ 0.17	1.02 $\pm$ 0.10	0.86 $\pm$ 0.08	1.13 $\pm$ 0.07
18	1	0.77 $\pm$ 0.08	0.76 $\pm$ 0.08	0.78 $\pm$ 0.07	0.93 $\pm$ 0.13

Results are the means  $\pm$  s.d. of two independent experiments. The ROS levels for the samples without any treatments are set as unity.

induction is actually caused by the scavenging effect of EGCG instead of that of DMSO. In this connection, the ROS level in directly irradiated CHO cells was measured under the treatment of adding the same concentrations of EGCG and DMSO as those in the previous experiment. Table 1 shows that the reduction in ROS levels by EGCG (1, 10 and 20  $\mu\text{M}$ ) in both irradiated and unirradiated samples are higher than that by DMSO. This result suggests that the reduction of MN formation in bystander cells is caused by the ROS scavenger EGCG.

In summary, the present study demonstrated that the signal transduction from irradiated cells to unirradiated cells occur within a short time after irradiation. EGCG scavenges ROS at a significant level in the bystander cells produced by alpha-particle irradiation, but not in directly irradiated cells.

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