Radiation Measurements 44 (2009) 1069–1072

Contents lists available at ScienceDirect

Radiation Measurements

journal homepage: www.elsevier.com/locate/radmeas

Study of influence of catechins on bystander responses in alpha-particle radiobiological experiments using thin PADC films

Y.L. Law, K.N. Yu*

Department of Physics and Materials Science, City University of Hong Kong, Tat Chee Avenue, Kowloon Tong, Hong Kong

ARTICLE INFO

Article history:
Received 14 October 2008
Received in revised form
25 May 2009
Accepted 18 October 2009

Keywords:
Bystander effect
Alpha-particle
Catechins
TUNEL

ABSTRACT

In this study, Chinese hamster ovary (CHO) cells were cultured in custom-made petri dishes with thin PADC films as substrates. Alpha particles with energies of 5 MeV were then irradiated from the bottom of PADC films. The DNA strand breaks in the bystander cells induced by irradiation were quantified with the use of terminal dUTP transferase-mediated nick end-labeling (TUNEL) assay. To study the influence of catechins on the bystander responses, catechins were added into the medium before alpha-particle irradiation of the cells. Fewer DNA strand breaks in the bystander cells were observed. As catechins are ROS (reactive oxygen species)-scavengers, the studied bystander cells might have been protected from radiation through scavenging of ROS by catechins.

1. Introduction

The induction of cell death, chromosome aberration and gene mutation by ionizing radiation have been widely studied (e.g., Kemp and Jeggo, 1986; Coates et al., 2004). Some of these biological effects are thought to result from production of reactive oxygen species (ROS), including oxygen ions, free radicals and peroxides. ROS are known to damage various cellular macromolecules including DNA, including double-strand breaks and single-strand breaks.

It has been long accepted that the DNA-damaging effects of ionizing radiation were caused by direct traversal of cell nuclei in the irradiated cells. However, an increasing amount of data from radiation studies has led to the proposal that damage signals can be transmitted from irradiated to unirradiated cells, leading to a variety of biological effects via a bystander effect. An earlier study showed that bystander effects could be induced even by a low dose of alpha particles, which led to the alpha-particle traversals of only 0.1-1% of cell nuclei (Nagasawa and Little, 1992). Bystander effect has been demonstrated for both high- and low-LET radiations but it is usually more significant for densely ionizing radiation such as alpha particles.

Although the underlying mechanisms of the bystander effect are not yet well understood, some evidence suggests that multiple signal transduction pathways can be involved. Oxidative metabolism (Azzam et al., 2002; Shao et al., 2003), gap-junction intercellular communication (GJIC) (Azzam et al., 2001; Ballarini et al., 2002) and secreted diffusible factors (Iyer et al., 2000; Shao et al., 2003) have been proposed to mediate the bystander effect.

Epigallocatechin gallate (EGCG) is the major component of the polyphenolic fraction of green tea. It makes up about 10-50% of the total green tea catechins with other catechins such as epicatechin (EC), epigallocatechin (EGC) and epicatechin gallate (ECG). Studies have revealed that EGCG is the most active component of catechins, 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). Studies have indicated that EGCG can play a role to protect against free-radical DNA damages in irradiated cells with low-LET radiations such as X-rays (Yang et al., 1994; Anderson et al., 2001).

In the present study, we aimed to study the influence of ECGC on alpha-particle induced bystander responses. In order to quantify the number of alpha-particle hits on the cell nuclei, a home made PADC-film cell dish was used to co-culture unirradiated and irradiated cells. Polyallyldiglycol carbonate (PADC) films are one of the most commonly used solid-state nuclear track detectors (SSNTDs) (see e.g., review by Nikezic and Yu 2004). There are distinct advantages of using PADC films as the cell-culture substrates in alpha-particle radiobiological experiments. For example, it is transparent, relatively biocompatible (Li et al. 2006), and does not dissolve in alcohol during sterilization (Chan et al., 2006). In the present experiments, cells were plated on both 22 and 1000 μm PADC films. The irradiated and unirradiated cells shared the medium during and after irradiation,

* Corresponding author. Tel. +852 2788 7812; fax: +852 2788 7830.
E-mail address: peter.yu@cityu.edu.hk (K.N. Yu).

1350-4487/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.radmeas.2009.10.030

but did not touch each other. This method was developed to study the radiation-induced bystander effects in a simpler system without the additional complication of GJIC. The effects of EGCG in the bystander cells were assessed using the TUNEL assay which characterizes DNA damages.

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_2 with MEM (Gibco): F-12 (Gibco) (1:1) mixed medium supplemented with 8% FCS (Gibco), 2×10^{-4} M Glycine (Sigma), 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco).

2.2. Alpha-particle irradiation

PADC films were used as the cell-culture substrates in this experiment. PADC films with a thickness of 22 μm were glued with epoxy (Araldite® Rapid, England) to the bottom of Petri dishes (Orange Scientific) with a diameter of 9 cm, with a hole of 1 cm diameter drilled at the bottom. A total of 8 pieces of PADC films with a thickness of 1000 μm and an area of 2 cm × 2 cm were put into the dishes to form the cell dishes as shown in Fig. 1. The cells plated on the 22 μm PADC film will be irradiated while the cells plated on the 1000 μm PADC film will be bystander cells.

The alpha-particle source employed was a planar ^{241}Am source (main alpha energy = 5.49 MeV under vacuum). Normal air was used as the energy absorber to control the alpha energies incident on the 22 μm PADC film. The cell dishes were irradiated from the bottom with 5 MeV alpha particles under normal incidence through a collimator for 3 min (Fig. 1). The residual energies of alpha particles that hit the cells were calculated by interpolation from the output of the SRIM program (Ziegler, 2003). After passing through the 22 μm PADC films, the residual energy of alpha particles hitting the cells is 1.62 MeV.

2.3. TUNEL assay

For the study of the influence of EGCG on the bystander responses, 2×10^7 cells from a confluent culture were seeded on the PADC film cell dish and EGCG (Sigma) was added to the culture medium from the stock solution (10 mM) in DMSO to give the appropriate final concentrations. After irradiation, the bystander cells on 1000 μm PADC films were transferred to another petri dish. The cells were trypsinized and pelleted by centrifugation. The supernatant was decanted and the cells were resuspended into 1 ml of ice-cold fixing solution (2% paraformaldehyde in PBS). The cells were incubated in fixing solutions for 1 h at room temperature.

At the end of the incubation, the cells were again pelleted and the fixing solution was removed. The pellets were then washed once in PBS and resuspended in 1 ml of ice-cold permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 10 min on ice. After permeabilisation, the cells were pelleted and washed twice in PBS. Negative control samples were treated in the same way but these were not irradiated, while a positive control sample was prepared by adding DNase I, grade I, to digest the DNA for 15 min at room temperature.

The cells in each sample were resuspended in 50 μl of TUNEL reaction mix from the in situ cell death detection kit, fluorescent, from Roche Diagnostics. This reaction mix includes 90% (v/v) Label solution and 10% (v/v) TUNEL enzyme. The reaction mixture was then incubated at 37 °C in dark for 1 h. The samples were washed twice in PBS and then resuspended in 1 ml of PBS for storage and analysis. The samples were analyzed by flow cytometer (Becton DICKINSON, FACSCalibur cytometer) by counting 10^4 cells for each sample to obtain the TUNEL signals.

2.4. Track formation and number of cell hits

After the bystander cells on the 1000 μm PADC films were transferred to another petri dish, the irradiated cells on the 22 μm PADC film were fixed with methanol:acetic acid (9:1 v/v). After air drying, the bottom sides of the cell dishes were etched by a 14 N KOH solution at 37 °C for 6 h. Such a low etching temperature was used to prevent the epoxy from dissolving in the strong etchant at high temperatures (Chan et al., 2008). As a result, alpha-particle tracks will be formed on the bottom of the PADC film cell dishes. The images of the cells and the tracks on the surface of 22 μm PADC film were captured. The number of cell hits can be obtained by counting the number of cell nuclei hit by 5 MeV alpha particles.

3. Results and discussions

3.1. Number of cell hits

The present investigation was designed to examine the induction of DNA damages in bystander cells by very low fluencies of alpha particles. An image of alpha-particle tracks with cell nuclei is shown in Fig. 2. From counting the number of cell hits, we found that as low as 1% (in a total of 10^7 cells) of the cell nuclei were actually traversed by alpha particles in all the samples. Table 1 shows the umber of cells

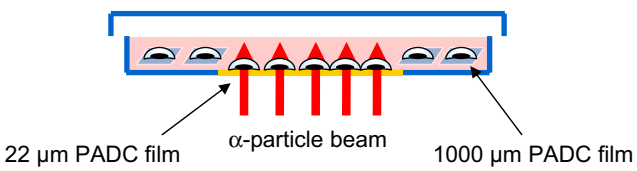

Fig. 1. Irradiation of a cell monolayer through the PADC film based cell dish.

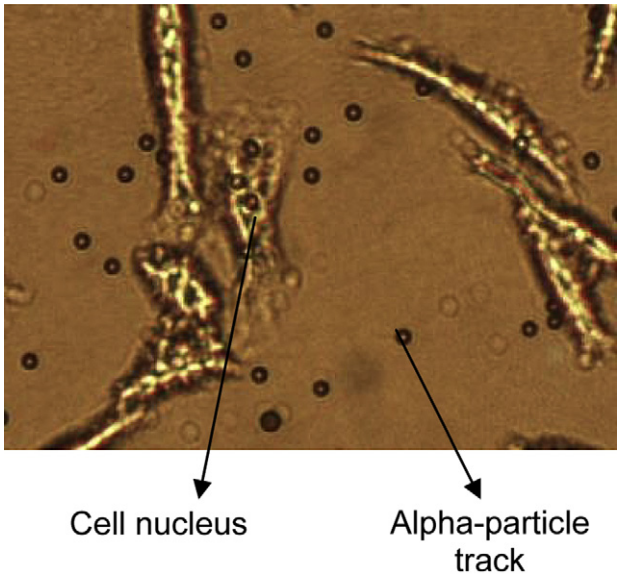

Fig. 2. Image of CHO cell nuclei together with 5 MeV alpha-particle tracks on the surface of a PADC film, captured with a magnification of 200x.

Table 1
Number of cells traversed by α particles and number of α particles per hit cell in different irradiated samples, as well as their relationship with the TUNEL positive signal in the corresponding bystander cell samples.

Concentration of EGCG (μM)	Mean number of cells traversed by α particles	Mean number of α particles that hit cells	Mean number of α particles per hit cell	Mean α particle energy received per cell (MeV)	Mean TUNEL signals (%)
0	229	303	1.32	2.14	15.16
1	236	368	1.56	2.53	3.57
10	252	313	1.24	2.01	12.85
20	138	243	1.76	2.85	12.85

traversed by α particles and the number of α particles per hit cell in different irradiated samples, as well as their relationship with the TUNEL positive signal in the corresponding bystander cell samples. The induction of DNA damages in bystander cells seems to be independent of the number of cells traversed by α particles or the number of α particles per hit cell. Even when as low as 1% (in a total of 10^7 cells) of the cells were actually traversed by alpha particles in the irradiated samples, the TUNEL positive signal of bystander cells could be as large as 15%. The results support that bystander damages occur even at very low doses and they do not increase significantly with dose (Ballarini et al., 2002). Further studies are needed to find out the relationship between the radiation dose and induction of DNA damages in bystander cells, if any, using different biological endpoints.

By using this PADC-film cell dish, the irradiated and unirradiated cells share the medium after irradiation but do not touch each other. We can avoid gap-junction communication, thus providing a useful approach to simplify the system and allowing us to focus on studies on mechanisms underlying medium-mediated bystander effects in future. Moreover, we can easily separate the bystander cells from the irradiated cells for different treatments. In this way, all results from the TUNEL assay are for the bystander cells only.

The images of the cells and the alpha-particle tracks on the PADC film were captured after the TUNEL analysis. Compared with the method proposed by Chan et al. (2008), the current method can avoid the complications from cell deaths during image capture before irradiation. Moreover, no more "base tracks" are needed to act as makers. As the procedure of chemical etching to generate base tracks can now be skipped, the roughness of the PADC film can be better controlled and the cells can be better seeded on the PADC film.

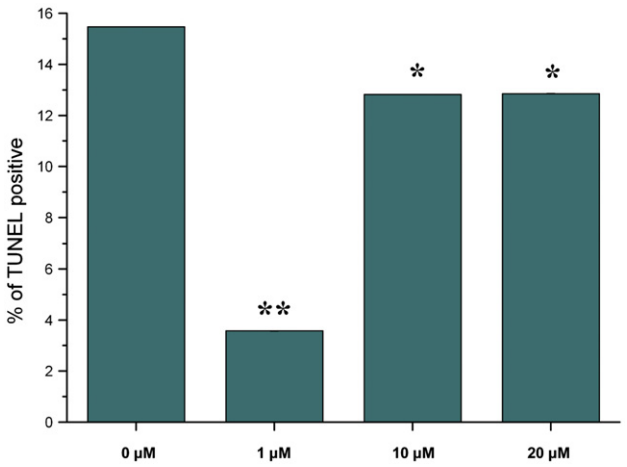

Fig. 3. Percentage of TUNEL positive in bystander CHO cells as a function of concentration of EGCG after irradiation. Results are means of three independent experiments (*$P < 0.05$, **$P < 0.005$, compared to the control, i.e., without any treatment). The error bars are too small to be shown in the figure.

3.2. Effects of EGCG on bystander cells

To investigate the effects of EGCG on induction of DNA damages in bystander cells, different concentrations of EGCG were added to the medium before irradiation. Immediately after irradiation, the bystander cells were fixed for the TUNEL assay. Fig. 3 shows the results. Here, TUNEL positive means that there are DNA strand breaks in the cell sample. The TUNEL results of all samples are given after adjustment for the corresponding unirradiated control. For example, if a particular bystander sample without any EGCG (0 μM) had 33.55% of signal and the corresponding control sample (unirradiated and without any EGCG) had 17.22% of signal, the bystander sample will have 16.33% (33.55%–17.22%) of TUNEL positive signal (Chan et al., 2008).

Fig. 3 shows the average results from three independent experiments. From Fig. 3, we can see that 15.47% of TUNEL positive signal occurs in bystander samples without any EGCG. Since the bystander cells were transferred to another petri dish for analysis immediately after irradiation, this result suggested that signal transduction from irradiated cells to bystander cells occurred within a short time and the signaling molecules secreted by the irradiated cells at early times after irradiation were crucial to the initiation of bystander responses.

When comparing the bystander samples without any treatment (0 μM -controls) to the samples with EGCG, the decreases in TUNEL positive signals were significant for concentrations of 1, 10 and 20 μM. The TUNEL positive signals in bystander cells were around 1.2-fold lower than the controls for 10 and 20 μM and 4.3- fold for 1 μM, with significant decrease ($P = 3.9 \times 10^{-4}$) for the case of 1 μM concentration. This is consistent with the results from a previous study showing that EGCG reduced the amount of prompt DNA strand breaks, at the physiologically significant concentrations of 1–2 μM (Anderson et al., 2001).

In our study, GJIC is not involved because the irradiated and unirradiated cells were spatially separated. This suggested that ROS might be involved in the induction of DNA breaks. From the mitigation of the bystander effects, the ROS scavenger EGCG is found to be active in reducing the amount of DNA breaks, which is likely due to the scavenging of the ROS before they react with the DNA of the bystander cells. Further studies are needed to identify the ROS involved in this system.

In summary, a PADC film cell dish was used in this study to successfully demonstrate the mitigation effects of EGCG on alpha-particle induced bystander effects in CHO cells.

References

Anderson, R.F., Fisher, L.J., Hara, Y., Harris, T., Mak, W.B., Melton, L.D., Packer, J.E., 2001. Green tea catechins partially protect DNA from OH radical-induced strand breaks and base damage through fast chemical repair of DNA radicals. Carcinogenesis 22, 1189–1193.

Azzam, E.I., de Toledo, S.M., Little, J.B., 2001. Direct evidence for the participation of gap-junction mediated intercellular communication in the transmission of damage signals from alpha-particle irradiated to non-irradiated cells. Proc. Natl. Acad. Sci. USA 98, 473–478.

Azzam, E.I., de Toledo, S.M., Spitz, D.R., Little, J.B., 2002. Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from alpha-particle-irradiated normal human fibroblast cultures. Cancer Res. 62, 5436–5442.

Ballarini, F., Biaggi, M., Ottolenghi, A., Sapora, O., 2002. Cellular communication and bystander effects: A critical review for modeling low-dose radiation action. Mutat. Res. 501, 1–12.

Chan, K.F., Siu, S.Y.M., McClella, K.E., Tse, A.K.W., Lau, B.M.F., Nikezic, D., Richardson, B.J., Lam, P.K.S., Fong, W.F., Yu, K.N., 2006. Alpha-particle radiobiological experiments using thin CR-39 detectors. Radiat. Protect. Dosim. 122, 160–162.

Chan, K.F., Yum, E.H.W., Wan, C.K., Fong, W.F., Yu, K.N., 2008. Study of DNA integrity in alpha-particle radiobiological experiments using thin CR-39 detectors. Radiat. Meas. 43 (Suppl. 1), S541–S545.

Coates, P.J., Lorimore, S.A., Wright, E.G., 2004. Damaging and protective cell signaling in the untargeted effects of ionizing radiation. Mutat. Res. 568, 5–20.

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

Kemp, L.M., Jeggo, P.A., 1986. Radiation-induced chromosome damage in X-ray sensitive mutants (xrs) of the Chinese hamster ovary cell line. Mutat. Res. 166, 255–262.

Li, W.Y., Chan, K.F., Tse, A.K.W., Fong, W.F., Yu, K.N., 2006. Studies of biocompatibility of chemically etched CR-39 SSNTDs in view of their applications in alpha-particle radiobiological experiments. Nucl. Instrum. Methods B 248, 319–323.

Nagasawa, H., Little, J.B., 1992. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Res. 52, 6394–6396.

Nikezic, D., Yu, K.N., 2004. Formation and growth of tracks in nuclear track materials. Mater. Sci. Eng. R 46, 51–123.

Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20, 933–956.

Shao, C., Aoki, M., Furusawa, Y., 2003. Bystander effect on cell growth stimulation in neoplastic HSGc cells induced by heavy-ion irradiation. Radiat. Environ. Biophys 42, 183–187.

Valcic, S., Burr, J.A., Timmermann, B.N., Liebler, D.C., 2000. Antioxidant chemistry of green tea catechins. New oxidation products of (−)-epigallocatechin gallate and (−)-epigallocatechin from their reactions with peroxyl radicals. Chem. Res. Toxicol. 13 (9), 801–810.

Yang, X.Q., Shen, S.R., Hou, J.W., Zhao, B.L., 1994. Mechanism of scavenging effects of (−)-epigallocatechin gallate of active oxygen free radicals. Acta Pharm. Sinica 14 (4), 350.

Ziegler, J.F., 2003. SRIM-2003.26. http://www.srim.org/.