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Studying effects of Magnolol on alpha-particle induced bystander effects using PADC-film based dishes

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ABSTRACT

Radiation-induced bystander effect refers to the biological response found in cells (called bystander cells) which are not irradiated directly by ionizing radiation but are next to cells irradiated directly by ionizing radiation. In the present paper, the effects of Magnolol, an extract from the bark of *Magnolia officinalis* which is used as a traditional Chinese medicine, were studied on alpha-particle induced bystander effects. In our experiments, Chinese hamster ovary (CHO) cells were cultured in PADC-film based dishes and were irradiated with low fluences of alpha particles passing through the PADC films. The precise number of cells traversed or missed by alpha particles could be determined by studying the alpha-particle tracks developed on the PADC films upon subsequent chemical etching. TdT-mediated dUTP Nick-End Labeling (TUNEL) assay was employed to analyze the biological response of bystander cells in terms of DNA strand breaks. With the pretreatment of Magnolol, the DNA strand breaks in bystander cells were reduced, which showed that the alpha-particle induced bystander effects were suppressed with the presence of Magnolol. Since Magnolol is an antioxidant which can scavenge reactive oxygen species (ROS), our results give support to that ROS play a role in the bystander signal transmission in our experiments.

1. Introduction

Alpha particles emitted from the naturally occurring short-lived radon progeny are the main contributor to the radiation dose derived from our natural background (e.g., Yu et al., 2006), and alpha-particle exposure has been shown to have a positive correlation with lung cancer incidence (e.g., Pavia et al., 2003). Radon risks (from radon progeny) can be enhanced by the so-called bystander effects. Radiation-induced bystander effects refer to the biological responses found in bystander cells which are not irradiated directly by an ionizing radiation but are next to cells irradiated directly by an ionizing radiation. Bystander responses include increased levels of sister chromatid exchange (Nagasawa and Little, 1992), DNA double strand breaks (Hu et al., 2005), micronucleus formation (Ponnaiya et al., 2004) and cell killing (Mothersill et al., 2004). Therefore, it is important to study the alpha-particle induced bystander effects and to propose ways to mitigate the associated elevated risks.

Various traditional Chinese medicines (TCMs) had been found effective to cure cancers. For example, the bark of *Magnolia officinalis* has an antitumor effect (Lin et al., 2001) and has been used as a traditional Chinese medicine for many years. Our current research aims at understanding the effects of TCMs on radiation effects. From our preliminary results, among a number of tested TCMs, Magnolol was shown to be the most effective in mitigating radiation-induced biological effects, so we decided to restrict our studies on Magnolol in the present paper. Magnolol, of which the chemical structure has been shown in Fig. 1, is a natural active component extracted from the bark of *Magnolia officinalis* (Lo et al., 1994), and is commonly used as a TCM.

We studied in the present paper whether Magnolol could mitigate the alpha-particle induced bystander effects in a cell co-culture system. TdT-mediated dUTP Nick-End Labeling (TUNEL) assay was used as a biological end point to detect DNA strand breaks in the bystander cells.

In the present studies, the number of cells hit by alpha particles needed to be known. Sufficiently thin polyallyldiglycol carbonate (PADC) films, a kind of commonly used solid-state nuclear track detectors which can record the tracks of alpha particles passing through it by subsequent chemical etching (see Nikezic and Yu (2004)

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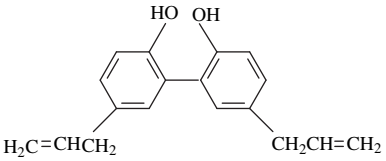

Fig. 1. Chemical structure of Magnolol.

for a review), were employed as cell culture substrates and to allow traversals of alpha particles to hit the cells (Chan et al., 2007). With the help of such PADC films, the precise number of cells hit by alpha particles was determined.

As Magnolol is also an antioxidant which can scavenge reactive oxygen species (ROS), the effects of Magnolol on radiation-induced bystander effects will also give evidence on whether ROS play a role in the bystander signal transmission in our experiments. ROS can be produced during the interaction between the radiation and the cell culture medium or the cells, and these reactive molecules can break DNA or chromosomes resulting in DNA fragmentation. A number of previous investigations showed that ROS were involved in the bystander signal transmission. A review of the relationship between ROS and bystander effect was given by Azzam et al. (2003).

2. Materials and method

2.1. Custom-made petri-dish

Custom-made petri-dishes were purposely fabricated for cell culture use in this experiment (Chan et al., 2007). Commercially available 100 μm thick PADC films (Page Mouldings Limited, Worcestershire) were etched to 22 μm with 1 N NaOH/Ethanol at 40 °C. A hole with a diameter of 10 mm was drilled at the bottom center of the 100 mm petri-dish, which was then covered by a 22 μm thick PADC film and fixed using epoxy on the bottom side. A total of 8 pieces of 2×2 cm^2 PADC films with 1 mm thickness were placed on the inner bottom of the petri-dish around the central hole Fig. 2. The custom-made petri-dishes were sterilized before use.

2.2. Preparation of Magnolol

Magnolol (Sigma) was dissolved in dimethyl sulfoxide (DMSO) with a concentration of 10 mM as the stock solution. The final working solution added to the cells was prepared by diluting this with the culture medium to no more than 0.2% DMSO.

2.3. Cell culture

Chinese hamster ovary (CHO) cells were routinely cultured in tissue culture flasks in Dulbecco modified Eagle's minimal essential

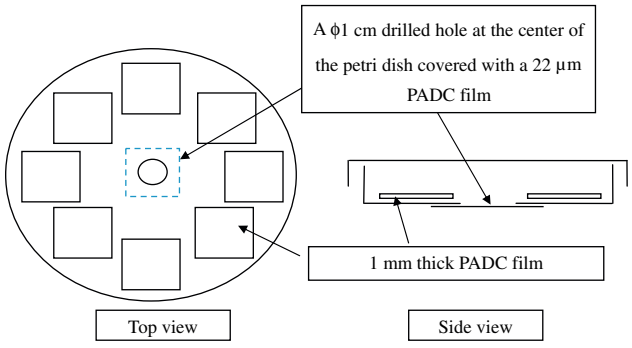

Fig. 2. A custom-made petri-dish.

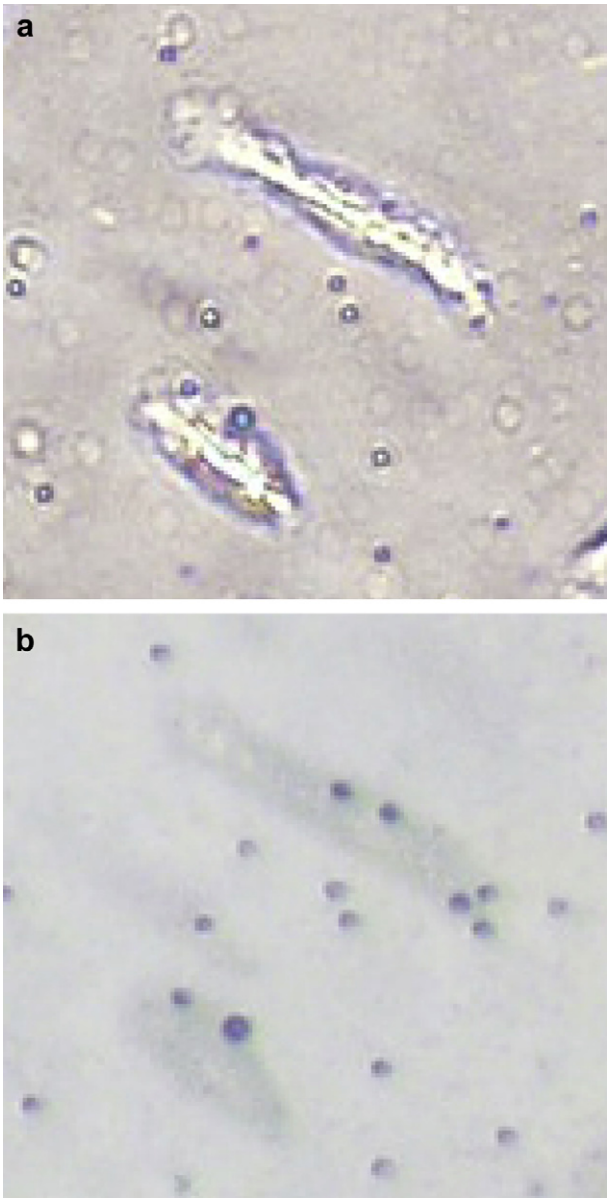

Fig. 3. Images of CHO cells on a 22 μm PADC film with alpha-particle tracks (dark spots) after chemical etching, obtained using an optical microscope from the side of the alpha-particle tracks: (a) in the transmission mode; (b) in the reflection mode.

medium (DMEM) with F12, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were incubated at 37 °C with humidified 5% CO_2. The cells were trypsinized by 0.5/0.2% (v/v) trypsin/EDTA and seeded with a density of 2.5×10^6 cells/dish in the custom-made petri-dishes. A volume of 20 ml cell culture medium was used in each dish to ensure that the medium could cover all the 1 mm thick PADC films. Different concentrations (1, 10 and 20 μM) of Magnolol were added 1 d prior to use for subsequent irradiation.

2.4. Alpha-particle irradiation

Confluent CHO cells were exposed to alpha particles from an ^{241}Am source from the side of the PADC-film base under normal incidence through a collimator. According to the SRIM program (Ziegler, 2006), the alpha particles have an energy of 1.62 MeV at the cell-PADC-film interface after passing through a 22 μm PADC film.

Table 1
Number of cells traversed by alpha particles and number of alpha 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 Magnolol (μM)	Mean number of cells traversed by alpha particles	Mean number of alpha particles that hit cells	Mean number of alpha particles per hit cell	Mean alpha particle energy received per cell (MeV)	Mean TUNEL signals (%)
0	551	792	1.44	2.33	33.23
1	412	504	1.22	1.97	19.82
10	341	493	1.45	2.35	26.64
20	351	433	1.23	1.99	20.07

2.5. TUNEL assay

After alpha-particle irradiation, the 1 mm thick PADC films were collected and the cells adhered on the films were trypsinized and transferred to a centrifuge tube immediately. The cells were then washed by phosphate buffered saline (PBS) and fixed by 2% formaldehyde in PBS for 1 h at room temperature. The cells were then rinsed by PBS again after fixation. Permeabilisation solution (0.1% Triton X-100 (Sigma) in 0.1% sodium citrate (Gibco)) was added to the cells for 10 min in a 4 °C refrigerator. The cells were then washed again with PBS two times. A positive control sample was prepared by adding DNase I for 15 min at room temperature before adding the TUNEL reaction mixture. The TUNEL reaction mixture (Roche Diagnostics GmbH, Germany) was made of 90% (v/v) Label solution and 10% (v/v) TUNEL enzyme was added to the cells and the cells were kept at 37 °C for 1 h. The cells were then washed twice in PBS. Analysis was performed by flow cytometry (Becton DICKINSON, FACSCalibur cytometry) which detected TUNEL signals in 10^4 cells from each sample with 450–500 nm excitation and 515–565 nm emission.

2.6. Track etching

Immediately after irradiation, the irradiated cells left on the 22 μm PADC films were rinsed by PBS and fixed using the fixation solution (90% methanol and 10% acetic acid (v/v)). The PADC films were then etched using 14 N KOH solutions at 37 °C for 6 hours to enlarge the alpha-particle tracks. The number of cells hit by alpha particles was then visually counted in the photos taken under the microscope.

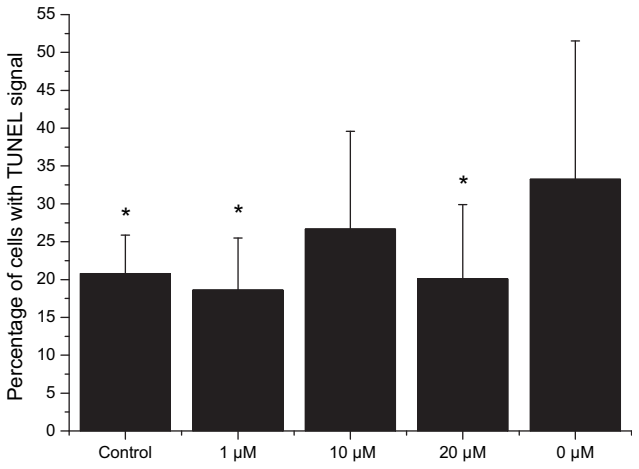

Fig. 4. Percentages of CHO cells with TUNEL signals for different samples. The non-irradiated sample is treated as the control. Comparing to the irradiated sample without any treatment of Magnolol, significant differences are observed in all samples treated with Magnolol except for the case of 10 M. (*$p < 0.05$).

2.7. Data analysis

The experiments were repeated at least 3 times with the data presented as the mean ± SD. Statistical analyses were performed using Student's *t*-test where cases with $P < 0.05$ were treated as statistically significant.

3. Results

3.1. Alpha-particle tracks and cell hits

The number of irradiated cells on the 22 μm PADC films was determined by counting the cells which overlapped with alpha-particle tracks, the latter being revealed upon chemical etching. Fig. 3 shows images of CHO cells and alpha-particle tracks obtained using an optical microscope in the transmission mode and reflection mode. From Fig. 3, it seems that the tracks are more clearly shown using the reflection mode and thus the number of cell hits is more accurately determined. Since the images were taken from the side of the alpha-particle tracks, some tracks may be covered by cells or water droplets in the transmission mode.

Table 1 shows the number of cells traversed by alpha particles and the number of alpha particles per hit cell in different irradiated samples, as well as their relationship with the TUNEL positive signal in the corresponding bystander cell samples. By visual counting, the number of cell hits, which was in the order of 10^2, was confirmed to be less than 1% of the total number of cells. Even for these low percentages of cells traversed by alpha particles, the TUNEL positive signal of bystander cells could range from about 20 to 30%. The energy of alpha particle was determined using the SRIM program as 1.62 MeV at the cell-PADC-film interface. Since the mean number of alpha particles incident on each cell was in the range from 1.22 to 1.45, each cell received about 2 MeV alpha-particle energy on average. The induction of DNA damages in bystander cells seems to be independent of the number of cells traversed by alpha particles or the number of alpha particles per hit cell.

3.2. Effect of Magnolol

The DNA strand breaks in the CHO cells were detected by flow cytometry with a sample size of 10^4 cells and the percentage of cells with TUNEL signals was then measured. As shown in Fig. 4, the mean percentage of bystander samples without pretreatment of Magnolol had about an extra 13% TUNEL signal when compared to the negative controls, even though only less than 1% CHO cells were hit by alpha particles, which clearly showed the presence of the bystander effect. Moreover, as the TUNEL assay was carried out immediately after the irradiation, the results also indicated that the bystander signal from irradiated cells to the bystander cells was present immediately after the irradiation. This was consistent with the results from another research group that the bystander signal was produced within a few minutes after irradiation (Hongying

et al., 2007). This also closely agreed with the ROS hypothesis as ROS were produced quickly in large amounts after alpha-particle irradiation and might eventually cause DNA strand breaks.

On the other hand, the bystander samples with pretreatment of 1 and 20 μM Magnolol gave TUNEL signals commensurate with that of the negative control but significantly different from the bystander sample without pretreatment of Magnolol. In other words, these two concentrations of Magnolol could mitigate the bystander effect. For the case of 10 μM, although the mean TUNEL signal was still lower than that of the untreated sample, the difference was not statistically significant.

4. Discussions and conclusion

Experimental results from previous researches suggested two types of mechanisms involved in the bystander effect. The first mechanism is the gap junction-mediated intercellular communication (GJIC), which is a cell-to-cell gap junction transfer route (Azzam et al., 2000), while the second one is the secretion of soluble factors from the irradiated cells into the medium (Narayanan et al., 1997). In the present experimental setup, since the CHO cells were not kept in touch with the others, the GJIC pathway was not considered. Our results indicated that the soluble factors from the irradiated cells were likely to involve ROS since the antioxidant Magnolol could mitigate the bystander effect. Moreover, by modifying the original experimental setup of Chan et al. (2007), the irradiated cells and the bystander cells could be spatially separated in the present experiments so they could also be separately studied. With the help of PADC films as cell culture substrates, information such as the precise number of cell hits or the energy of alpha particles incident on the cells could be determined. The extra information could help us know more about the mechanisms of the bystander effect. With the success of the present experiments, different biological end points or different potential TCMs can be studied with the aid of PADC films in future.

As mentioned before, the bark of *Magnolia officinalis* has been used as a traditional Chinese medicine for many years and has an antitumor effect (Lin et al., 2001). Therefore, Magnolol may act as ideal radiation protector to protect us from radiation-induced bystander effects especially during radiotherapy against tumors. Moreover, as the ROS scavenging properties of Magnolol can successfully mitigate the bystander effect, other TCMs which have similar antioxidant properties may also have the capability in mitigating radiation-induced bystander effects.

In conclusion, radiation-induced bystander effects in CHO cells in terms of DNA strand breaks were mitigated with pretreatment of 1 and 20 μM Magnolol. Magnolol with a concentration of 10 μM also showed some effects but these were not statistically significant.

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