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Influence of Magnolol on the bystander effect induced by alpha-particle irradiation

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ABSTRACT

In this work, the influence of Magnolol on the bystander effect in alpha-particle irradiated Chinese hamster ovary (CHO) cells was examined. The bystander effect was studied through medium transfer experiments. Cytokinesis-block micronucleus (CBMN) assay was performed to quantify the chromosome damage induced by alpha-particle irradiation. Our results showed that the alpha-particle induced micronuclei (MN) frequencies were suppressed with the presence of Magnolol.

1. Introduction

Alpha particles emitted from radon and radon progeny contribute the main part of the natural background radiation. It is also known that alpha-particle exposure has a positive correlation with lung cancer incidence (e.g., Pavia et al., 2003). However, the exact mechanisms behind the alpha-particle induced carcinogenesis are still not fully understood. It has long been assumed that the direct interaction between ionizing radiation and a cell is required to trigger a biological effect. However, evidence accumulated over 17 years has indicated that some biological responses can occur in cells that have not been irradiated directly by ionizing radiation but are next to irradiated cells. These responses include increased level of sister chromatid exchange (Nagasawa and Little, 1992; Lehnert and Goodwin, 1997), DNA double strand break (Hu et al., 2005), mutations (Huo et al., 2001), micronucleus formation (Ponnaiya et al., 2004), cell cycle arrest (Azzam et al., 2000) and cell killing (Mothersill et al., 2004). This phenomenon is known as the bystander effect. The bystander effect has changed our knowledge of ionizing radiation and we need to reconsider the way we estimate the health risk of ionizing radiation.

The bystander effect is hypothesized to be induced through secreting soluble factors from the irradiated cells into the medium (Narayanan et al., 1997), or through gap junction-mediated intercellular communication (GJIC) which is a cell to cell gap junction transfer route (Azzam et al., 2001). Although the exact mechanisms of bystander effects remain to be elucidated, reactive oxygen species (ROS) are thought to be potentially involved in the bystander signal transmission (Azzam et al., 2003). These oxidants consist of different chemical species such as superoxide anions, hydrogen peroxide and hydroxyl radicals. ROS are highly reactive and their targets include the DNA, and DNA breaks or chromosome breaks would occur in environments with high oxidative stress.

Magnolol (Fig. 1) is a natural active component with strong antioxidant properties extracted from the bark of *Magnolia officinalis* (Lo et al., 1994; Shen et al., 1998), and the bark of *Magnolia officinalis* is commonly used in traditional Chinese medicine and Japanese remedies. Apart from its antioxidant properties, it has lots of pharmacological effects including an anti-tumor capacity (Lin et al., 2001), anti-platelet aggregation (Pyo et al., 2002), anti-fungal (Bang et al., 2000), anti-bacterial and anti-inflammatory effects (Park et al., 2004). However, its radiation protective effects have not been studied. In the present work, we have investigated the ROS scavenging properties of Magnolol and its capability in suppressing alpha-particle induced bystander effects through the medium transfer approach. Micronucleus (MN) formation, which is a hallmark of genetic aberration, would be formed in un-repaired chromosome breaks due to ionizing radiation. MN assay is a reliable, simple and rapid chromosome aberration assay, which can be applied to examine the formation of MN in cells. Chinese hamster ovary (CHO) cells were used in the present experiments since they are particularly suitable for the *in vitro* micronucleus assay (Aardema et al., 2006).

2. Materials and methods

2.1. Cell culture

CHO cells were routinely cultured in tissue culture flasks in Dulbecco modified Eagle's minimal essential medium (DMEM) with F12, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and

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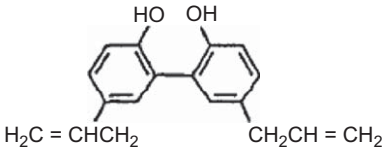

Fig. 1. Molecular structure of Magnolol.

100 mg/ml streptomycin. The cells were incubated at 37 °C with humidified 5% CO_2. The cells were trypsinized and seeded in 60 mm petri-dishes which have a 10 mm hole drilled at the center and glued with a Mylar film of 3.5 μm thickness. These cells were seeded with a density of 2×10^4 cells/dish one day prior to use for subsequent irradiation, and these will form our irradiated samples. For bystander cells, cells were seeded in 35 mm petri-dishes with a density of 1.2×10^4 cells/dish one day prior to use.

2.2. Preparation of Magnolol

Magnolol (Sigma) was dissolved in dimethyl sulfoxide (DMSO) with a concentration of 20 mM as the stock solution. The final working solution added to the cells was prepared by diluting with the culture medium to no more than 0.1% DMSO.

2.3. Alpha-particle irradiation

Just before alpha-particle irradiation, all culture media were removed and fresh media were added. Confluent CHO cells were exposed to alpha particles from a ^{241}Am source with a dose of 18 cGy from the side of the Mylar-film base. The alpha particles have an average energy of ~5.2 MeV at the cell-Mylar-film interface delivered at a dose rate of 0.3 cGy/s.

2.4. CBMN assay

After alpha-particle irradiation, the media which held the irradiated cells were collected and filtered with a 0.2 μm filter to remove all cells and supernatants in the media. The filtered media were added with 2.5 μg/ml Cytochalasin B which blocked the cells at the cytokinesis stage and then transferred to the bystander cells in the 35 mm petri-dishes. Fresh media were also added to the irradiated cells together with 2.5 μg/ml Cytochalasin B. Both the irradiated and bystander cells were then returned to the incubator for 24 h. The cells were then washed by PBS twice and fixed using the fixation solution (90% methanol and 10% acetic acid) for 15 min. The fixation solution was then removed and 0.02% Acridine orange (AO) was added for 5 min. After 5 min, the AO solution was washed away and the samples were dried for one day. Observations were made under the fluorescent microscope with a thin layer of distilled water on the cells and covered with a glass cover. The scoring criteria of MN of Fenech et al. (2003) were followed. The number of micronuclei in at least 500 binucleate (BN) cells was counted.

2.5. Data analysis

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

3. Results

3.1. Time dependence of micronucleus induction in bystander cells

To study the time effects on MN induction in bystander cells, confluent CHO cells were exposed to alpha particles with a dose of 18 cGy, and then returned to the 37 °C incubator for 0.5, 1 and 3 h before medium transfer, or transferred immediately after alpha-particle irradiation. MN assay was performed at 24 h after the medium transfer. Fig. 2 shows the variation of micronucleus induction in non-irradiated bystander cells with different incubation time of irradiated cells. Comparing to the non-irradiated controls, the highest percentage of excessive MN formation in the bystander cells occurred when the bystander cells received the medium from irradiated cells immediately after alpha-particle irradiation. Interestingly, the level of MN induction dropped as the post-irradiated incubation time increased. This suggests that the MN-inducing bystander signals induced by alpha-particle irradiation was present immediately after irradiation and had a short life time. These data have also been reported separately by Law et al. (2008).

3.2. Effect of Magnolol on micronucleus induction in bystander cells

In order to examine whether Magnolol can suppress the alpha-particle induced bystander effect, different concentrations of Magnolol (1, 10 and 20 μM) were added 3 h before alpha-particle irradiation and during medium transfer. The effect of 0.1% DMSO was also investigated in this study. Medium transfer was performed immediately after alpha-particle irradiation as suggested from the previous experimental results in which the maximum MN-inducing activities occurred at that time point. Sham-irradiation samples were treated as controls. As shown in Fig. 3, all tested concentrations of Magnolol have no toxic effects on CHO cells. It can significantly reduce the MN induction level in the bystander cells. About 40%, 30% and 30% of mean MN induction levels in bystander cells were suppressed by 1, 10 and 20 μM Magnolol treatment, respectively, when compared to sham-treated samples. Moreover, 0.1% DMSO can also suppress the MN induction level in bystander cells, however, with a smaller suppressing effect comparing to the case of Magnolol. The 0.1%

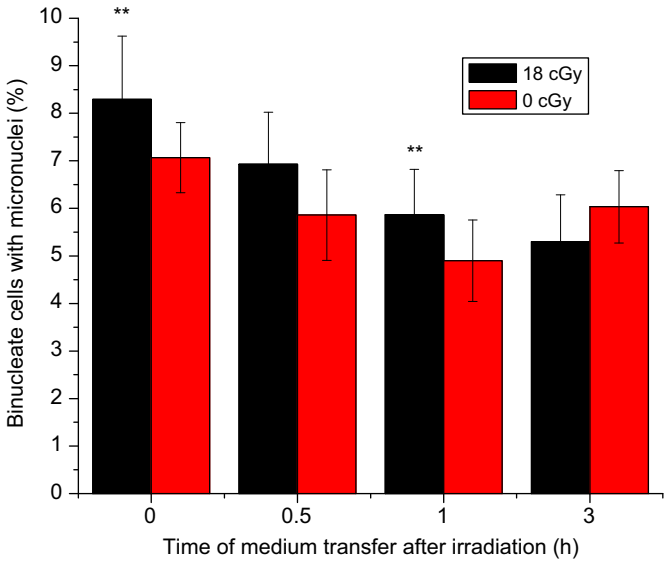

Fig. 2. MN induction level in bystander cells as a function of the time of medium transfer after irradiation. Results are presented as the mean ± SD of three independent experiments. (**$p < 0.1$ obtained from t-tests).

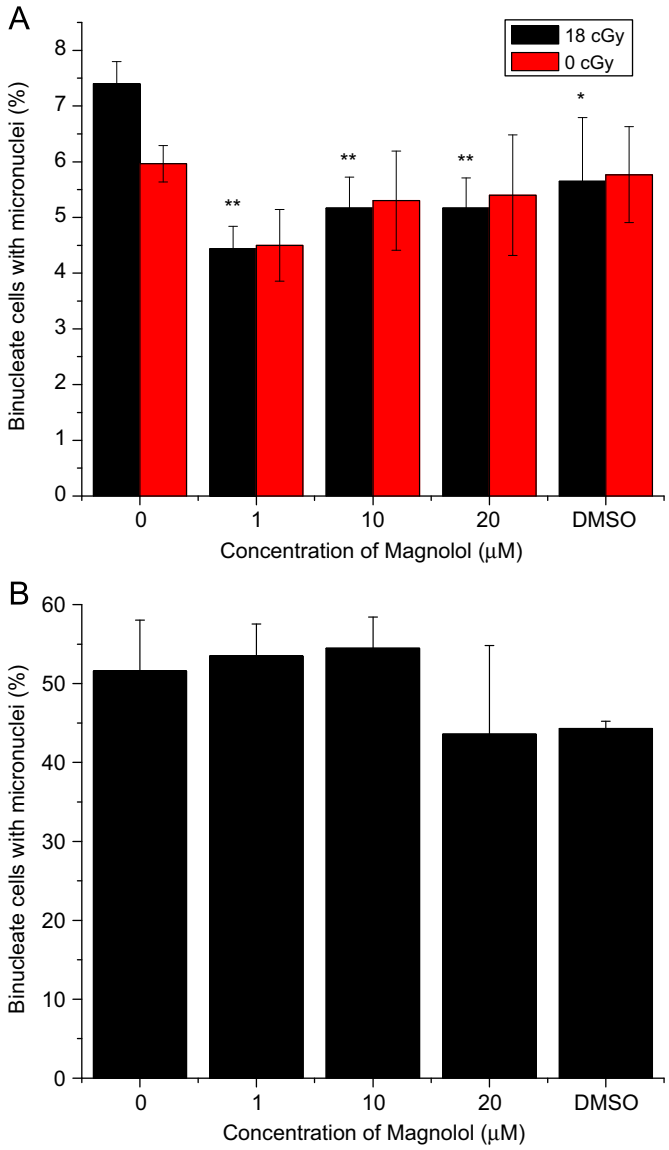

Fig. 3. Effect of different concentrations of Magnolol on induced micronuclei in bystander cells (A) and irradiated cells (B) after 18 cGy alpha-particle irradiation. The results show the mean number of percentage of MN ± SD in 500 binucleate cells from three independent experiments. A significant difference is only observed between sham-treated bystander cells and Magnolol or 0.1% DMSO treated bystander cells ($^{*}p < 0.05$, $^{**}p < 0.0005$ obtained from t-test).

DMSO can only suppress by about 24% the mean MN induction levels in bystander cells. Fig. 3 shows the level of MN induction in irradiated cells. Unlike the case for bystander cells, all tested concentrations of Magnolol as well as 0.1% DMSO do not show significant suppressing effects on the MN induction level in irradiated cells.

4. Discussion

Previous experiments have shown that the biological effects caused by ionizing radiation can be independent of direct traversals to the cells. In this study we found that alpha particles can induce excessive MN formation in non-irradiated CHO cells through medium transfer (Fig. 2). It is suggested that some bystander signals were initiated by the interactions between alpha particles and the cells or the medium, and then these factors were transferred to the bystander cells through the medium. Lehnert and Goodwin (1997) reported that alpha particles could induce two types of bystander signals, namely, those from interactions between alpha particles and serum-containing culture medium or alpha-irradiated cells.

In the present work, the bystander signal is found to be maximum when the medium was transferred immediately from the irradiated cells after alpha-particle irradiation. However, the MN induction level decreased within a period as short as 30 min suggesting these MN-inducing bystander signals were not stable and had a short life time, and thus they could not persist for a long time. The result is consistent with the previous experimental results from other research groups. For example, Lehnert and Goodwin (1997) found that SCE-inducing factor by alpha particles lost its activity after exposure to alpha-particles. In addition, Yang et al. (2007) also demonstrated that high linear energy transfer (LET) iron-ion irradiation elicited MN induction in normal human skin fibroblasts and the bystander signals could only persist for 3 h and then decreased.

The main purpose of this study is to test whether Magnolol, the active component widely found in Traditional Chinese Medicine (TCM) that has strong antioxidant effects, can protect cells from direct alpha-particle irradiation and the bystander effects. Our results show that concentrations of 1, 10 and 20 μM Magnolol can effectively suppress MN induction in bystander cells thus protecting their chromosomes from damage (Fig. 3A). This suggests the involvement of ROS in the MN-inducing bystander effect, and is also consistent with the results from previous investigations. Many previous experiments showed that bystander effects were suppressed with the use of a ROS scavenger, suggesting the participation of ROS as an important role in bystander effects (Azzam et al., 2003). For example, alpha particles were found to be able to initiate biological production of superoxide anions and hydrogen peroxide, which can be inhibited by addition of superoxidase dismutase (SOD) (Narayanan et al., 1997). The two phenolic groups of Magnolol give it the property of free radical scavengers (Taira et al., 1993). As a result, Magnolol might react with those ROS and terminate the chain reactions of ROS, and thus MN formation in bystander cells would decrease with the treatment of Magnolol. In addition, Magnolol can inhibit nuclear factor-κB (NF-κB) activation which is a transcription factor (Tse et al., 2007), and inhibition of NF-κB activation was found to significantly decrease the bystander effect (Zhou et al., 2008; Azzam et al., 2002). This provides another possible pathway by which Magnolol suppresses the bystander effect.

Interestingly, among all tested concentrations of Magnolol, the 1 μM Magnolol has the strongest power of suppressing the bystander effect. A recent experiment showing that an antioxidant may act as a prooxidant if the concentration is high (Park and Lee, 2008). This may explain why the suppression of the bystander effect by 10 and 20 μM Magnolol would be weaker. DMSO is also an antioxidant and has been reported to suppress bystander signals (Bishayee et al., 2001). As Magnolol was dissolved in DMSO, the effect of the highest concentration of DMSO we used in this experiment, i.e., 0.1% DMSO, was also investigated. Our result shows that it can also inhibit the excessive MN induction level in bystander cells, but the suppression was not as strong as that of Magnolol. It can only suppress about 24% mean MN induction levels in bystander cells while Magnolol can decrease about 30–40%. This proves that Magnolol did prevent excessive MN formation in bystander cells. Although ROS was suggested to be involving in the medium-mediated bystander effect as shown by our results, how these ROS ultimately cause MN formation remains unknown. Further investigations are needed to examine the mechanism of the bystander effect.

Fig. 3B shows that Magnolol can significantly reduce the MN induction level in bystander cells but not in the irradiated cells. This is also true in the case of 0.1% DMSO. One possible explanation is that the damage mechanism in irradiated cells and bystander cells are different. Alpha particles cause direct damages on irradiated cells, and thus Magnolol cannot function on irradiated cells by its antioxidant properties. Another possibility is that the MN induction level by direct alpha-particle irradiation is too high. Previous experiments found that a single alpha particle could induce tens of thousands of ROS when it traversed through a mammalian cell (Feinendegen, 2002). Therefore, even if a small fraction of cells can be protected by Magnolol, we cannot observe the effects of Magnolol in irradiated cells.

The presence of bystander effects has far-reaching implications on the risk assessment of ionizing radiation, such as background natural radiation and the medical use of radiation. We might have underestimated the risk if we do not take into account the bystander effects of ionizing radiation. When we come to discuss the protective effects of an antioxidant on mitigating the bystander effect, toxicity of the antioxidant is an important consideration. In our experiments, Magnolol did not show any toxic effect on the CHO cells at the tested concentrations. Moreover, ''*Houpu*'', from which the natural active component Magnolol is extracted, has historically been used in Chinese medicine for a long time. It has a very low genotoxitity and is thus safe for dietary consumption (Li et al., 2008). Therefore, Magnolol provides a cheap and safe way to protect us form radiation induced bystander effects. In addition, Magnolol has also been found to have an antitumor effect (Lin et al., 2001). The antitumor effect together with the ability to suppress radiation-induced bystander effect may mean that Magnolol has a very important role in radiotherapy against tumors.

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