Inhibitory effects of linarin on the IR-induced cancer cell migration and invasion in human non-small-cell lung cancer A549.

Ah-Reum Han [a], Hong-Duck Um [b], Chan-Hun Jung [c*]
[a]*Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup-si, Jeollabuk-do 56212, Republic of Korea*
[b]*Division of Applied Radiation Bioscience, Korea Institute of Radiological & Medical Sciences, Seoul 01812, Republic of Korea*
[c]*Jaseng Spine and Joint Research Institute, Jaseng Medical Foundation, Seoul 06110, Republic of Korea*
[*]*Corresponding author: chjung@jaseng.co.kr*

1. Introduction

Radiotherapy is routinely used in the treatment of lung cancer patients. However, it often causes malignant effects, such as promoting cancer cell migration and invasion [1,2]. Previous studies demonstrated that ionizing radiation (IR) promotes cancer cell invasion by stimulating the β-catenin, IL-6, STAT3, and Bcl-XL signaling pathway [3] or the PI3K, Akt, and NF-κB signaling pathway [4]. Both Bcl-XL and NF-κB stimulate the secretion of matrix metalloproteases (MMPs), including MMP-2 and MMP-9 [4,5].

The dried flowers of *Chrysanthemum morifolium* Ramat. (Compositeae) have been used in a traditional medicine to treat inflammation, arteriosclerosis, and hypertension [6]. In previous phytochemical studies on *C. morifolium*, flavonoids and phenolic acids have been identified as major constituents [7], which have exhibited diverse biological activities such as antioxidant [8], anti-inflammatory [9], and antitumor [10] effects. Numerous varieties and cultivars have also been developed by hybridization and mutation for horticultural purposes [11]. Recent gamma-irradiated mutation breeding studies on the chrysanthemum cultivar 'Noble Wine' resulted in the development of 'ARTI-Dark Chocolate (ADC)' (registration No. 4996) (Fig. 1(A)) [12]. Several flavonoids were isolated from ADC flowers, including linarin (Fig. 1(B)) [13]. Linarin has been reported to have anti-inflammatory [14], anti-hypertensive [15], and anticancer [16] effects. However, the potential inhibitory effects of linarin against IR-induced cancer cell migration and invasion have not been examined.

In this study, we investigated the inhibitory effects of linarin on the IR-induced cancer cell migration and invasion using human non-small-cell lung cancer A549 cells.

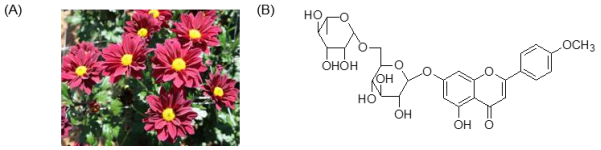

Fig. 1. (A) The flowers of a gamma-irradiated mutant cultivar, 'ARTI-Dark Chocolate', (B) Chemical structure of linarin.

2. Methods and Results

2.1 Cell viability of linarin

The cytotoxic effects of linarin against the non-small-cell lung cancer A549 cell line were determined by the MTT assay, upon treatment with various concentrations of linarin (5, 10, 50, 100, 200, and 500 μM). The results of the 24-h MTT assays showed that linarin had effect on cell viability with an IC50 value of 282 μM. Since linarin showed cell viability of 94.1 ± 5.7% at a concentration of 5 μM, this concentration was used in the next experiments.

2.2 Linarin inhibits IR-induced migration and cell invasion

To examine the effect of 5 μM linarin on the migration of γ-irradiated A549 cells, wound healing assays were performed for monitoring cell motility. As shown in Fig. 2, γ-irradiated A549 cells migrated more across the wound area, leading to approximately 80% healing at 48 h, compared with about 50% healing in the same time for non-irradiated cells. The treatment of linarin suppressed wound healing of γ-irradiated cells to the level of non-irradiated cells. The invasiveness of A549 cells was measured as their ability to invade a Matrigel barrier in an invasion assay. While the invasiveness of A549 cells was induced by IR at a dose of 10 Gy, this ability considerably decreased in linarin-treated γ-irradiated A549 cells (Fig. 3).

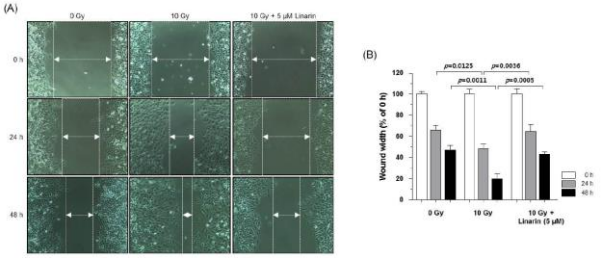

Fig. 2. Effect of linarin on the IR-induced migration of A549 cells. (A) Wound healing assay was performed to examine the effects of linarin treatment on the IR-induced migration of A549 cells. (B) Relative wound width was calculated as the ratio of the remaining wound width at the given time point and the original width at 0 h. Data represent the mean ± SD (n = 3).

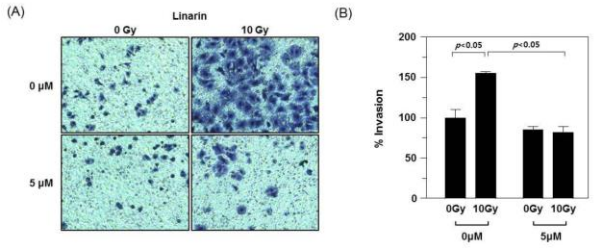

Fig. 3. Effect of linarin on the IR-induced invasion of A549 cells. (A) Cell invasion was performed using Matrigel-coating-transwell in non-irradiated and γ-irradiated A549 cells treated with 5 μM linarin. After 24 h incubation, cell invasiveness was compared on Matrigel-coated polycarbonate filters with or without 5 μM linarin. (B) The percentage of invasion was analysed and represented as cells number per field of cell invasion and compared with each non-irradiated A549 cell. Data represent the mean ± SD (n = 3).

2.3 Linarin does not inhibit β-catenin/STAT3/Bcl-XL/Src pathway

To investigate whether linarin suppressed the IR-mediated induction of cellular pathways involving β-catenin, STAT3, and Bcl-XL, Western blotting was performed. Data revealed that linarin did not attenuate the activation of β-catenin, STAT3, and Bcl-XL (Fig. 4(A)). Because Bcl-XL overexpression promotes cell invasion by stimulating the Src phosphorylation signaling pathway [5], we further examined whether linarin modulated Src phosphorylation and found that Src phosphorylation was not suppressed by linarin (Fig. 4(B)). Together, these results demonstrated that linarin did not inhibit the IR-mediated activation of the β-catenin/STAT3/Bcl-XL/Src pathway.

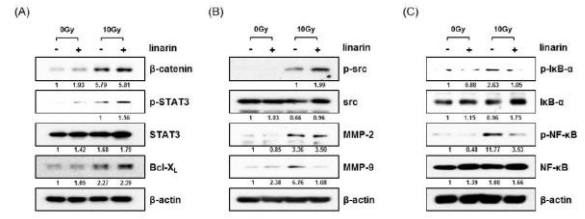

Fig. 4. Linarin inhibits the IR-induced MMP-9 expression by blocking the NF-κB activity. A549 cells were irradiated with 10 Gy of γ-rays and then 5 μM linarin was added. After 24 h incubation, incubated cells were recovered and cell lysates were prepared. The levels of (A) β-catenin, phosphorylated STAT3 (p-STAT3), STAT3, and Bcl-XL, (B) phosphorylated Src (p-src), Src, MMP-2, and MMP-9, and (C) phosphorylated Iκ-Bα (p-Iκ-Bα), Iκ-Bα, phosphorylated NF-κB(p-NF-κB), and NF-κB were compared by Western blotting in A549 cells that were non-irradiated (0 Gy) or irradiated (10 Gy) with γ-rays and treated with or without 5 μM linarin. β-actin was used as a loading control.

2.4 Linarin inhibits MMP-9 but not MMP-2 expression

MMP-2 and MMP-9 are closely related to cancer cell metastasis, invasion, and angiogenesis [17]. Therefore, we investigated the effect of linarin on MMP-2 and MMP-9 expression. Immunoblotting experiments showed that MMP-2 and MMP-9 expression was higher in irradiated cells than in non-irradiated cells (Fig. 4(B)). MMP-9 expression was downregulated in cells treated with linarin (Fig. 4(B)), whereas MMP-2 expression showed no differences in irradiated cells treated with or without linarin. These data suggest that linarin specifically inhibits MMP-9 expression in irradiated cells.

2.5 Linarin inhibits NF-κB and IkB-α phosphorylation

To investigate whether MMP-9 downregulation in the presence of linarin (Fig. 4(B)) was due to NF-κB downregulation, we examined the expression levels of NF-κB and its phosphorylated forms by Western blotting. Because the activation of NF-κB signaling is preceded by the degradation of the inhibitory subunit IkB-α [18], the expression levels of IκB-α and its phosphorylated form were also investigated. NF-κB subunits (p65 and/or p50) are normally sequestered in the cytosol as an inactive complex by binding to inhibitory factor IκB-α in unstimulated cells. Upon stimulation by signals including IR, IκB-α is phosphorylated by IKK and inactivated through ubiquitin-mediated degradation. The resulting free NF-κB is translocated into the nucleus and acts as a transcription factor [18]. Gamma-irradiation of A549 cells increased the expression of phosphorylated NF-κB; however, NF-κB phosphorylation was markedly inhibited following linarin treatment (Fig. 4(C)). Moreover, the accumulation of phosphorylated IkB-α was higher in irradiated cells than in non-irradiated cells and was accompanied by the degradation of IkB-α. These observations suggest that linarin suppresses NF-κB activation.

3. Conclusions

Our results demonstrated that linarin inhibited the IR-induced cancer cell migration and invasion, by downregulating MMP-9 expression via the suppression of NF-κB activation in human non-small-cell lung cancer A549 cells. Therefore, linarin may be effective in countering the undesirable effects of IR in human lung cancer and improving the therapeutic effects of radiotherapy.

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