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Radiosensitizing effect of combined triptolide and irradiation treatment in lung cancer cell lines

M. Kong[1], J.W. Lee[2], M. Yun[3], S.H. Lee[4*]

[1]Division of Lung/Head and Neck Oncology, Department of Radiation Oncology, Kyung Hee University Medical Center, Kyung Hee University School of Medicine, Seoul, Republic of Korea
[2]Medical Science Research Institute, Kyung Hee University Medical Center, Seoul, Republic of Korea
[3]Department of Bioindustry & Bioresource Engineering, College of Life Sciences, Sejong University, Seoul, Republic of Korea
[4]Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Kyung Hee University Medical Center, Kyung Hee University School of Medicine, Seoul, Republic of Korea

ABSTRACT

Background: No study has reported radiosensitizing effect of triptolide in lung cancer cell lines. We explored the effect and underlying molecular mechanisms of combined triptolide and irradiation treatment in lung cancer cell lines. ***Materials and Methods***: Colony formation assays were conducted to test the radiosensitizing effect of triptolide in A549 and H460 lung cancer cell lines. Survival fractions and sensitizing enhancement ratios were calculated. To determine the underlying mechanism of triptolide and irradiation combination, immunofluorescence cytometric analysis of apoptosis was conducted after treatment with triptolide and/or 4 Gy irradiation. To explore the molecular mechanisms of apoptosis induced by triptolide and irradiation combined treatment, western blot analysis was conducted after treatment with triptolide and/or irradiation (1, 2, 3, or 4 Gy). The antibodies used for Western blotting were PARP, JNK, p53, HSP70, and Akt. ***Results:*** Combined triptolide and irradiation treatment significantly decreased the surviving fractions than irradiation alone in both cell lines. Triptolide and irradiation combination treatment also resulted in significant increase in apoptosis rates than irradiation alone in both cell lines. The expression of PARP cleavage, JNK, and p53 were prominent in the groups treated with triptolide and irradiation combination. The expression of HSP70 and Akt were suppressed in groups treated with the triptolide and irradiation combination. ***Conclusion:*** This study showed that triptolide in combination with irradiation enhanced antitumor effects in lung cancer cell lines.

Keywords: *Radiotherapy, lung cancer, triptolide, radiation sensitizing agent.*

► ***Original article***

Corresponding authors:
Seung Hyeun Lee, M.D., Ph.D.,
Fax: *+ 82 2 958 2848*
E-mail:
humanmd04@daum.net

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INTRODUCTION

Lung cancer is one of the most lethal malignant tumors in Korea and other countries in the world [1,2]. Radiation treatment (RT) is the standard treatment for patients with locally advanced lung cancer. Although many clinical studies have reported promising RT treatment outcomes in patients with locally advanced lung cancer in recent years [3-6], radio-resistance is considered a major obstacle to the success of RT. Therefore, new therapeutic agents to enhance the effectiveness of RT are needed.

Currently, there is growing interest in the therapeutic applications of bioflavonoids for the treatment of cancers. Triptolide is a diterpenoid triepoxide derived from the Chinese herb *Tripterygium wilfordii* that has been used as a natural medicine in East Asia for hundreds of years. Triptolide is reported to have various

pharmacologic effects including anti-inflammation[7,8], anti-oxidant [8,9], and anti-cancer activities[10-13]. In addition, it was found that when triptolide was combined with chemotherapeutic agents, increased synergistic cytotoxic effects were found in many cancer cell lines [14-18]. Furthermore, several studies reported that triptolide in combination with ionizing radiation produced synergistic anti-tumor effects in pancreatic cancer, nasopharyngeal carcinoma, and oral cavity cancer both in vitro and *in-vivo* [19-21]. However, no study has reported the radiosensitizing effect of triptolide in lung cancer. To find new radiosensitizing agent to enhance the effectiveness of RT in lung cancer, we hypothesized that a triptolide and irradiation combination treatment would also increase radiosensitivity in lung cancer. In this study, we explored the effect and underlying molecular mechanisms of combined triptolide and irradiation treatment in lung cancer cell lines.

MATERIALS AND METHODS

Human lung cancer cell lines A549 and H460 were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). The cells were cultured in RPMI-1640 (Corning Life Science, Tewksbury, MA, USA) containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Atlas Biologicals, Fort Collins, CO, USA), in culture dishes at 37°C in a humidified atmosphere with 5% CO_2. Subculture and media changes were performed once every 2-3 days. Triptolide (purity ≥98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Triptolide stock solution was stored in DMSO at -20°C. Irradiation was performed at room temperature with a linear accelerator (Clinac iX, Varian Medical System, Palo Alto, CA, USA) at a dose rate of 2.0 Gy/min.

To test the radiosensitizing effect of triptolide in A549 and H460 human lung cancer cell lines, colony formation assays were conducted. A549 and H460 cells were seeded in 60 mm culture dishes (2.5×10^5 per dish). After incubation for 24 hours, cells were treated with irradiation alone (1, 2, 3, or 4 Gy) or combination of triptolide (75 nM for A549 and 50 nM for H460 cells) and irradiation (1, 2, 3, or 4 Gy). Triptolide was administered immediately before irradiation and maintained for 2 hours, followed by trypsinization and cell counting. Cells were cultured in 6-well plates at different densities according to irradiation dose and incubated at 37°C for 7 days to allow for colony formation. After 7 days, colonies were fixed and stained with 1% Gentian violet. The colonies with >50 cells were scored as surviving colonies. Plating efficiency was calculated as dividing the average number of colonies per dish by the amount of cells plated. Survival fractions were calculated as values normalized to the plating efficiency of appropriate control groups. Sensitizing enhancement ratios were calculated according to the D_0 values using the following formula: D_0 of irradiation alone treated cells/D_0 of triptolide and irradiation combination treated cells.

To determine the underlying mechanisms of the radiosensitizing effect of triptolide, immunofluorescence cytometric analysis of apoptosis was conducted. Twenty-four hours after treatment with triptolide (75 nM for 549 and 50 nM for H460 cell lines) and/or 4 Gy irradiation, cells were harvested, washed with cold phosphate-buffered saline and resuspended in binding buffer at a concentration of 1×10^6 cells/mL. Both 5 μL of annexin V/FITC and 5 μL of propidium iodide (PI) per 10^5 cells were added and incubated for 15 minutes in the dark at room temperature. After incubation, flow-cytometric analysis was conducted according to the manufacturer's protocols (BD FACSCalibur Flow Cytometer, BD Biosciences, San Jose, CA, USA). CellQuest Pro software (BD Biosciences, San Jose, CA, USA) was used to analyze the data and the percentage of cells that were annexin V-positive but PI-negative was compared among the different treatment groups.

Western blot analyses were conducted to explore the molecular mechanisms of apoptosis induced by combined triptolide and irradiation treatment. Twenty-four hours after treatment with triptolide (75 nM for 549 and 50 nM for

H460 cell lines) and/or irradiation (1, 2, 3, or 4 Gy), the cells were lysed in ice-cold cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA), collected and homogenized, and then centrifuged at 12,000 rpm for 10 minutes. The protein concentrations were quantified using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein were electrophoresed through 8-12% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk or 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) at room temperature. After blocking, the membranes were washed and incubated overnight with specific primary antibodies at 4°C. The antibodies used for Western blotting were PARP, Jun N-terminal kinase (JNK), p53, 70-kDa heat shock protein (HSP70), Akt, and β-actin (Cell Signaling Technology, Danvers, MA, USA). Antibody of β-actin was used as an endogenous control. The membranes were washed and incubated with horseradish-peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Then, the membranes were processed with enhanced chemiluminescence and scanned using an Amersham Imager 600 (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). The relative concentration values of each blot were calculated and analyzed using the Multi Gauge program.

All experiments were conducted at least in triplicate. SPSS version 20.0 was used for statistical analysis. Independent t-tests or one-way analysis of variance were used to analyze the significance between groups. A P-value of <0.05 was considered statistically significant.

RESULTS

The radiosensitizing effect of triptolide in combination with irradiation in the A549 lung cancer cell line was evaluated. Seven days after irradiation, the surviving fractions were 91%, 65%, 48%, and 33% for 1, 2, 3, and 4 Gy, respectively. However, combination of triptolide with irradiation significantly decreased the clonogenicity. Seven days after combined triptolide (75 nM) and irradiation treatment, the surviving fractions were 77%, 51%, 34%, and 24% for 1, 2, 3, and 4 Gy, respectively (P<0.05, figure 1A). The radiosensitizing effect of triptolide was also evaluated in the H460 lung cancer cell line. Seven days after irradiation, the surviving fractions were 93%, 60%, 37%, and 19% for 1, 2, 3, and 4 Gy, respectively. However, combination triptolide with irradiation again significantly decreased the surviving fractions. Seven days after irradiation with triptolide (50 nM), the surviving fractions were 69%, 44%, 27%, and 12% for 1, 2, 3, and 4 Gy, respectively (P<0.05, figure 1B). The sensitizing enhancement ratios of triptolide were 1.56 in the A549 and 1.51 in the H460 cell lines. These results indicate that triptolide has a potential radiosensitizing effect in human lung cancer cell lines.

To test whether the triptolide and irradiation combination enhances cell apoptosis in lung cancer cell lines, ongoing apoptosis was detected by annexin V staining. In the A549 lung cancer cell line, the apoptosis rates were 7.01% in the control group (no treatment), 9.46% in the groups treated with 4 Gy irradiation, 30.12% in the groups treated with triptolide (75 nM), and 40.82% in the groups treated with irradiation and triptolide combination (figure 2). Apoptosis rates were significantly higher in the groups treated with the triptolide and irradiation combination than other groups (P<0.05). Annexin V staining results of the H460 cell line were similar to those of the A549 cell line. In the H460 cell line, the apoptosis rates were 7.29% in the control groups, 15.10% in the groups treated with irradiation, 39.23% in the groups treated with triptolide (50 nM), and 50.58% in the groups treated with both irradiation and triptolide (figure 3). In the H460 cell line, triptolide and irradiation combination also resulted in a significant increase in apoptosis rate than in the other treatment groups (P<0.05).

To explore the molecular mechanism of the radiosensitizing effect of triptolide, several key

apoptotic molecules were evaluated by Western blot analyses. In the A549 lung cancer cell line, the expression of PARP cleavage, JNK, and p53, which are important molecules that play critical roles in apoptotic pathways, were prominent in the groups treated with the triptolide (75 nM) and irradiation combination relative to the groups treated with irradiation alone (figure 4A, B and C). On the contrary, the expression of HSP70 and Akt, molecules that are involved in apoptosis inhibition, were suppressed in the groups treated with the triptolide and irradiation combination relative to the groups treated with irradiation alone (figure 4D and E). The molecular mechanisms of triptolide were also evaluated in the H460 lung cancer cell line, and the results were similar to those of the A549 cell line. The expression of PARP cleavage, JNK, and p53 were prominent in the groups treated with the triptolide (50 nM) and irradiation combination than the groups treated with irradiation alone (figure 5A, B and C). On the contrary, the expression of HSP70 and Akt were suppressed in the groups treated with combination therapy than the groups treated with irradiation alone (figure 5D and E).

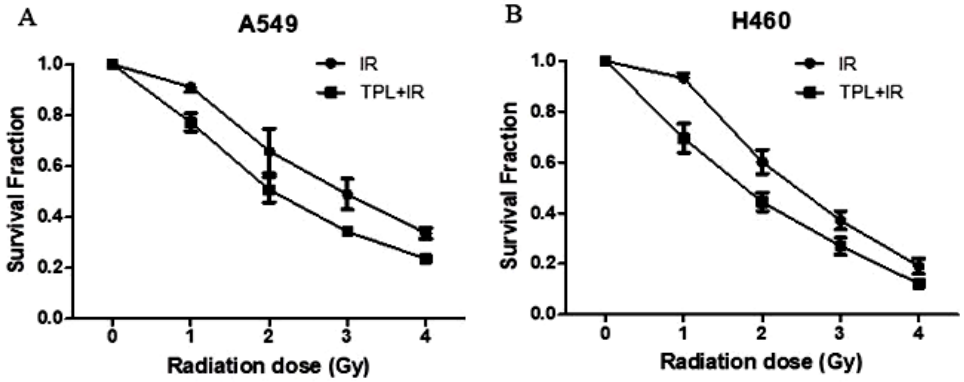

Figure 1. The radiosensitizing effect of triptolide (TPL) in combination with irradiation (IR) in lung cancer cell lines. A549 cells were treated with 75 nM triptolide and/or indicated doses of radiation. H460 cells were treated with 50 nM triptolide and/or indicated doses of radiation. Triptolide was administered immediately before irradiation and maintained for 2 hours. (A) Surviving fractions were measured by colony formation assay in the A549 lung cancer cell line. (B) Surviving fractions were measured by colony formation assay in the H460 lung cancer cell line. The data shown are the average of triplicate experiments.

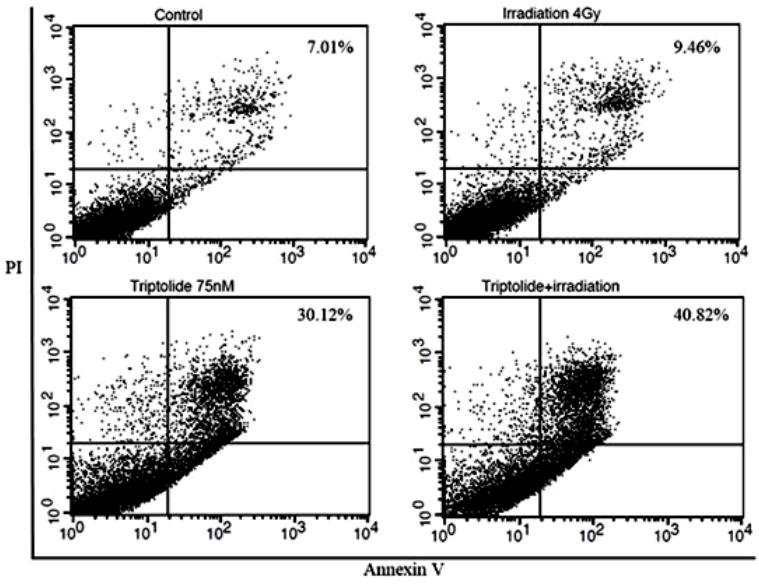

Figure 2. Ongoing apoptosis was detected by annexin V staining in the A549 lung cancer cell line. Apoptosis rates were significantly higher in the group treated with the triptolide and irradiation combinaion than other groups (P<0.05).

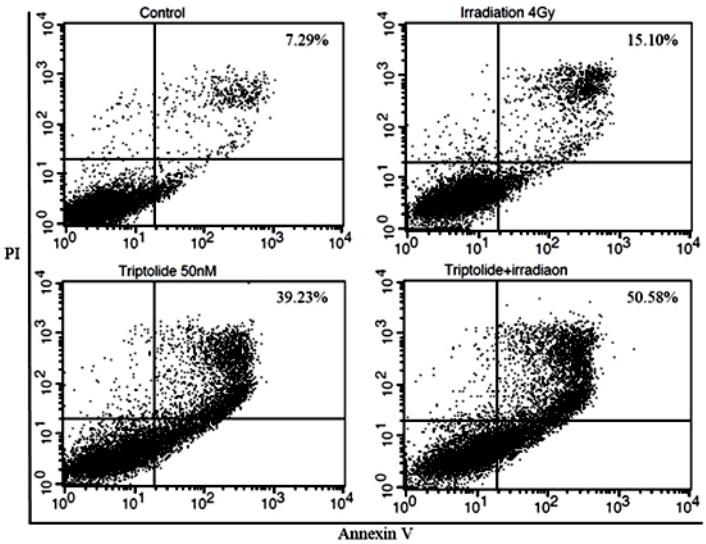

Figure 3. Ongoing apoptosis was detected by annexin V staining in the H460 lung cancer cell line. Apoptosis rates were significantly higher in the group treated with the triptolide and irradiation combination than other groups (P<0.05).

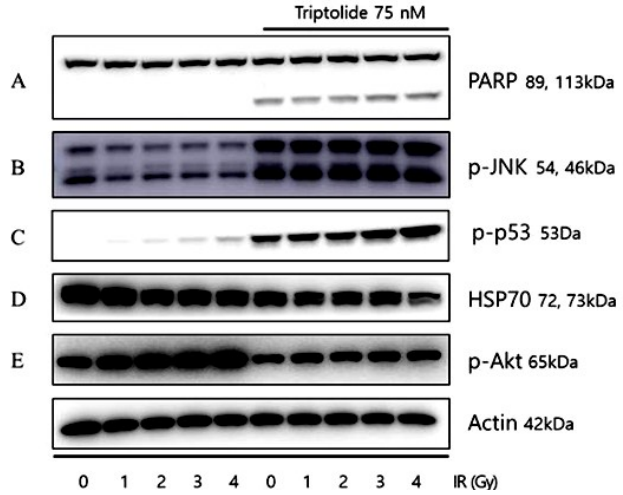

Figure 4. Molecular mechanisms of the triptolide radiosensitizing effect in the A549 lung cancer cell line. The expression of (A) PARP cleavage, (B) JNK, and (C) p53 were prominent in the groups treated with the triptolide (75 nM) and irradiation combination than the groups treated with irradiation alone. The expression of (D) HSP70 and (E) Akt were suppressed in the groups treated with combination therapy than the groups treated with irradiation alone.

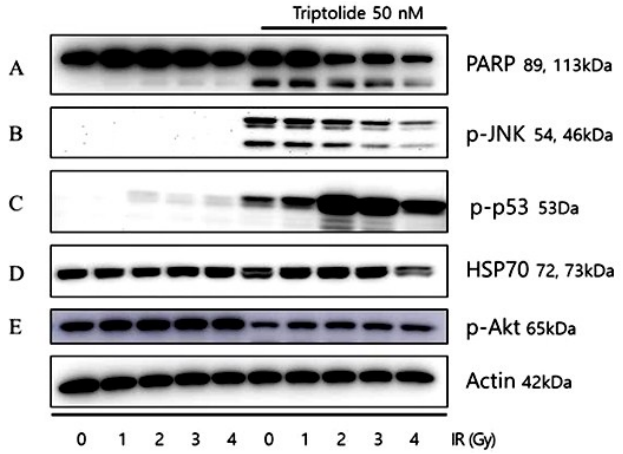

Figure 5. Molecular mechanisms of the triptolide radiosensitizing effect in the H460 lung cancer cell line. The expression of (A) PARP cleavage, (B) JNK, and (C) p53 were prominent in the groups treated with the triptolide (50 nM) and irradiation combination than the groups treated with irradiation alone. The expression of (D) HSP70 and (E) Akt were suppressed in the groups treated with combination therapy than the groups treated with irradiation alone.

DISCUSSION

Previous studies reported that triptolide showed radiosensitizing effects in oral cancer, pancreatic cancer, and nasopharyngeal cancer cell lines [19,20,22]. In addition, several studies reported that triptolide enhanced chemosensitivity in several cancer cell lines [7,23-27]. However, as far as we know, no study has reported the radiosensitizing effect of triptolide in lung cancer cell lines. This is the first study to evaluate the effect and underlying molecular mechanisms of triptolide treatment combined with irradiation in lung cancer cell lines. In this study, we conducted colony formation assays to test the radiosensitizing effect of triptolide in

A549 and H460 human lung cancer cell lines, and found that the triptolide and irradiation combination significantly decreased the surviving fractions compared with irradiation alone (figure 1). It is well known that radiation-induced apoptosis may be used to predict radiosensitivity in several cancer cell lines, and increased apoptosis rate means that the cancer cells have higher radiosensitivity [22,23,28,29]. In our study, apoptosis rates were significantly higher in the groups treated with the triptolide and irradiation combination than in the groups treated with irradiation alone (figure 2 and 3). These results also indicated that triptolide enhanced apoptosis induction when combined with irradiation and increased the radiosensitivity in human lung cancer cell lines.

Although we identified some molecules involved in apoptotic pathways that were prominent in the lung cancer cell lines treated with the triptolide and irradiation combination, the exact molecular mechanisms underlying the radiosensitizing effect of triptolide in lung cancer cell lines are remains unclear. Wang et al. reported that the radiosensitizing effect of triptolide is associated with the mitochondria-dependent apoptotic pathway in a pancreatic cancer cell line [19]. Zhang *et al.* also reported that Bcl-2 family proteins, located on the mitochondrial membrane, play important roles in the radiosensitizing effect of triptolide in a nasopharyngeal cancer cell line [20]. In our study, we also identified PARP cleavage, a downstream apoptotic exertive molecule of the mitochondria-dependent apoptotic pathway, was highly activated in the combination triptolide and irradiation groups than those treated with irradiation alone (figure 4 and 5). To confirm whether the radiosensitizing effect of triptolide is associated with the mitochondria -dependent apoptotic pathway in lung cancer cell lines, we have plan to evaluate the expression of Bcl-2 family proteins, cytochrome c, caspase-8, and caspase-9 in lung cancer cell lines treated with triptolide and irradiation combination in our subsequent studies.

Chen *et al.* reported that the triptolide and irradiation combination significantly increased the proportion of cells in the G2-M phase, which is the most radiosensitive portion of the cell cycle, in an oral cancer cell line [21]. It is well known that cell cycle phase is an important factor in cell radiosensitivity. However, the effect of the triptolide and irradiation combination treatment on cell cycle has not been studied in lung cancer cell lines. In this study, we also evaluated the effect of triptolide on cell cycle and we found that the combination triptolide and irradiation treatment slightly increased the proportion of H460 cancer cells in the G2-M phase ($P>0.05$). However, the combination treatment did not increase the proportion of A549 cells in the G2-M phase (data not shown). We are conducting additional studies to explore the effect of triptolide combined with irradiation on cell cycle in lung cancer cell lines.

There are some concerns with respect to the use of radiosensitizers in cancer treatment. One of the major concerns is that radiosensitizers might increase normal tissue toxicities [24]. However, several studies reported no significant weight loss in mice treated with triptolide and irradiation, and these results suggested that the combination triptolide and irradiation treatment does not induce obvious toxicity [19,20]. In addition, triptolide is now in phase II trials for arthritis treatment with tolerable toxicity, and has been used in nephritic syndrome, idiopathic pulmonary fibrosis, and rheumatoid arthritis without obvious toxicities [7,25]. Therefore, we believe that triptolide can be safely used in lung cancer patients who treated with RT. In vivo animal studies and clinical studies for lung cancer patients will be necessary to confirm the safety of the triptolide and irradiation combination treatment in lung cancer.

In conclusion, this study showed that triptolide in combination with irradiation enhanced antitumor effects in lung cancer cell lines. These results suggest that triptolide may be a promising candidate of radiosensitizer for the treatment of lung cancer.

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***Conflicts of interest*: Declared none.**

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