

Ginsenoside Rg3 sensitizes human non-small cell lung cancer cells to γ -radiation by targeting the nuclear factor- κ B pathway

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Abstract. At present, it is elusive how non-small cell lung cancer (NSCLC) develops resistance to γ -radiation; however, the transcription factor nuclear factor- κ B (NF- κ B) and NF- κ B-regulated gene products have been proposed as mediators. Ginsenoside Rg3 is a steroidal saponin, which was isolated from *Panax ginseng*. Ginsenoside Rg3 possesses high pharmacological activity and has previously been shown to suppress NF- κ B activation in various types of tumor cell. Therefore, the present study aimed to determine whether Rg3 could suppress NF- κ B activation in NSCLC cells and sensitize NSCLC to γ -radiation, using an NSCLC cell line and NSCLC xenograft. A clone formation assay and lung tumor xenograft experiment were used to assess the radiosensitizing effects of ginsenoside Rg3. NF- κ B/inhibitor of NF- κ B (I κ B) modulation was ascertained using an electrophoretic mobility shift assay and western blot analysis. NF- κ B-regulated gene products were monitored by western blot analysis. The present study demonstrated that ginsenoside Rg3 was able to sensitize A549 and H1299 lung carcinoma cells to γ -radiation and significantly enhance the efficacy of radiation therapy in C57BL/6 mice bearing a Lewis lung carcinoma cell xenograft tumor. Furthermore, ginsenoside Rg3 suppressed NF- κ B activation, phosphorylation of I κ B protein and expression of NF- κ B-regulated gene products (cyclin D1, c-myc, B-cell lymphoma 2, cyclooxygenase-2, matrix metalloproteinase-9

and vascular endothelial growth factor), a number of which were induced by radiation therapy and mediate radioresistance. In conclusion, the results of the present study suggested that ginsenoside Rg3 may potentiate the antitumor effects of radiation therapy in NSCLC by suppressing NF- κ B activity and NF- κ B-regulated gene products, leading to the inhibition of tumor progression.

Introduction

Lung cancer is the leading cause of cancer-associated mortality and was responsible for 160,340 estimated mortalities in the United States in 2012, >80% of which were diagnosed as non-small cell lung cancer (NSCLC) (1). The treatment for NSCLC includes stage-dependent therapy, surgery, radiotherapy and chemotherapy. In addition, adjuvant methods, including molecular-targeted therapy, are vital choices (2). As a therapeutic strategy for the treatment of NSCLC, irradiation is the first choice under many circumstances. However, only ~20% of patients achieve complete pathological responses to irradiation, due to problems with radioresistance and toxicity (3). Efforts to improve this rate have focused on overcoming the resistance of NSCLC to radiation therapy by increasing the radiation dose, or by using radiosensitizers that may decrease its toxic effect (4); however, currently neither of these approaches has resulted in significantly improved outcomes.

Certain types of Chinese drugs have been shown to provide a rich resource for the identification of anticancer drugs. Previous and ongoing studies by our group focused on observing the anticancer activity of ginseng and related Chinese medicines. The roots of *Panax ginseng* have been used in the treatment of various disorders (5). Keum *et al* (6) previously reported that in HL-60 human pro-myelocytic leukemia cells, the antitumor effects of ginsenoside Rg3 are caused by inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced activation of nuclear factor κ B (NF- κ B) (6). Since NF- κ B inhibitors have radiosensitizing potential (7), the present study hypothesized that ginsenoside Rg3 may also radiosensitize NSCLC cells.

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In the present study, the hypothesis that ginsenoside Rg3 may be able to radiosensitize NSCLC cells was investigated by measuring the effects of ginsenoside Rg3 on the growth of cultured NSCLC cells and NSCLC xenografts in C57BL/6 mice, both of which were exposed to radiation. The present study demonstrated that ginsenoside Rg3 sensitized NSCLC to radiation by down-regulating NF- κ B-regulated gene products, leading to inhibition of tumor progression.

Materials and methods

Materials and cell culture. Raw ginseng (Jilin province, China) was steamed at 120°C using an autoclave (Techeng Machinery Equipment Ltd., Shanghai, China) for 2 h; the Rg3 content was then measured using high performance liquid chromatography, as previously reported by Kim *et al* (8). Primary polyclonal antibodies against inhibitor of NF- κ B (I κ B; sc-371), phosphorylated (p)-I κ B (sc-52943), matrix metalloproteinase-9 (MMP-9; sc-10737), cyclooxygenase-2 (COX-2; sc-23984), vascular endothelial growth factor (VEGF; sc-7269), B-cell lymphoma 2 (Bcl-2; sc-492) and β -actin (sc-8432) were diluted according to the manufacturer's instructions and were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Primary polyclonal antibodies against cyclin D (#2922) and c-myc (#9402) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated secondary antibodies (1:4,000) were obtained from Santa Cruz Biotechnology, Inc. Parental A549 and H1299 human lung carcinoma cells, and the Lewis lung carcinoma cell line (LLC) were obtained from the Cell Center of the Chinese Academy of Medical Sciences (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco Life Technologies (Carlsbad, CA, USA). All of the cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells in the exponential growth phase were used for subsequent experiments. The NF- κ B Electrophoretic Mobility Shift assay (EMSA) kit was obtained from Viagene Biotech (Ningbo, China).

Stable cell lines were generated by transfecting A549 cells with the pNF- κ B-TA-Luc reporter construct (Beyotime Institute of Biotechnology, Haimen, China) using GeneJuice® transfection reagent (Novagen®; EMD Millipore, Billerica, MA, USA), according to the manufacturer's instructions. The plasmid pNF- κ B-TA-Luc contains the luciferase gene sequence driven by an artificial promoter element with four NF- κ B binding sites. Cells with genomic incorporation were selected on the basis of antibiotic resistance (geneticin; Invitrogen Life Technologies, Carlsbad, CA, USA). The newly generated cell line A549-pN- κ B-TA-Luc was cultured in DMEM supplemented with geneticin (800 μ g/ml) and was split (1:5) every seven days. The medium was refreshed after a growth period of six days. Lipopolysaccharide (1 μ g/ml; Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 4 h at room temperature. Luciferase activity was then examined using a Luciferase Assay kit (Promega Corp., Madison, WI, USA) to evaluate the reporter response.

Clonogenic survival assay. A549 and H1299 cells were treated with vehicle (acetone; 2 μ M) or 25 μ M ginsenoside Rg3 for

24 h and were then irradiated with a single dose of 0, 1, 2, 4, 6, 8 and 10 Gy using a ⁶⁰Co unit (FHC50; Shanghai Medical Instrument Factory, Shanghai, China) at room temperature. Ginsenoside Rg3 was rinsed away by washing cells in sterile saline over 3 h, and the cells were trypsinized and replated in 60-mm Petri dishes at an appropriate cell density: 0 Gy, 40/ml; 1 Gy, 60/ml; 2 Gy, 1.5x10²/ml; 4 Gy, 3.0x10²/ml; 6 Gy, 8.0x10²/ml; 8 Gy, 1.6x10³/ml; 10 Gy, 5.0x10³/ml. Colony formation was achieved by incubation for 10-14 days. At the end of the experiment the cells were stained with Giemsa (Beyotime Institute of Biotechnology) and the colonies were counted. The plating efficiency (PE) and survival fraction (SF) were calculated using the following equations: PE=(colony number/inoculating cell number) x100%; SF=PE (tested group)/PE (0 Gy group) x100%. A dose-survival curve was obtained for each experiment and used for calculating survival parameters. Parallel samples were set at each radiation dosage. The survival curve was plotted using Origin 7.5 software (OriginLab Corporation, Northampton, MA, USA), using the following equation: SF=1-(1-e^{-D/D₀})^N. The cellular radiosensitivity (mean lethal dose, D₀), the capacity for sublethal damage repair (quasi-threshold dose, Dq) and the extrapolation number (N) were calculated according to the multitarget, single-hit model. The D₀ values were used to calculate sensitizer enhancement ratios (SER) (9).

In vivo antitumor effects of ginsenoside Rg3 with or without radiation. Six-week-old male C57BL/6 mice provided by Zhengzhou University (Zhengzhou, China) were used in the present study. All experiments were approved by the Ethical Committee of Zhengzhou University Health Science Center (Zhengzhou, China). The mice were maintained in a climate-controlled room at 20°C with a 12 h light/dark cycle and had free access to standard mice chow and water. A total of 0.2 ml LLC cells (1x10⁷ cells/ml) were injected subcutaneously into the right hind leg of C57BL/6 mice. Tumor volume was determined using caliper measurements of the tumor length (L) and width (W), according to the following formula: Tumor volume=0.5236x W² x L (10). Treatment was initiated when the tumors in each group had reached an average volume of 200 mm³, at ~6 days post inoculation. The mice were randomly assigned to the following treatment groups (n=8): Untreated controls; irradiation only; ginsenoside Rg3 only (10 mg/kg twice per week, orally); and combination of ginsenoside Rg3 and irradiation. Mice in the control and radiation-only groups were administered with the acetone vehicle. Tumors in the legs were exposed to 8 Gy of γ -radiation using a ⁶⁰Co irradiator at a rate of 1 Gy/min immediately following treatment with the drug. The time for the tumor volume to increase by five baseline values was calculated for each mouse. The median times and standard errors were calculated for each group.

EMSA. Protein (15 μ g) was extracted from cell nuclei using a nuclear extraction kit (Viagene Biotech); the protein was then prepared, stored and qualified by methods described previously (11). No reducing agents were added to the EMSAs. EMSA was performed using a DNA-Protein Binding Detection kit (Viagene Biotech), according to the manufacturer's instructions. Briefly, an NF- κ B oligonucleotide

probe (5'-AGTTGAGGGGACTTCCAGGC-3') was labeled with biotin. The binding reaction was carried out in a 10- μ l mixture containing 1 μ l 10X incubation buffer, 1 μ l poly(deoxyinosinic-deoxycytidylic) and nuclear extracts. Following a 20-min incubation at room temperature, 0.5 μ l biotin-labeled probe was added to the samples. Following a further incubation for 20 min at room temperature, the samples were separated by 6% non-denaturing polyacrylamide gel (Applygen Technologies, Inc., Beijing, China), at 150 V for 2 h at 4°C. Subsequently, the gel was dried and exposed to X-ray film (Kodak, Tokyo, Japan).

Assay of NF- κ B transcription/promoter activity. A total of 5×10^4 viable A549-pNF- κ B-TA-Luc cells were seeded into each well of a 24-well plate. After 12 h, the cells were exposed to ^{60}Co radiation (8 Gy) and were then harvested 3 or 6 h later. In the combination group, the cells were pretreated with 25 μM ginsenoside Rg3 24 h prior to irradiation. Luciferase activity was examined using a Luciferase Assay kit (Promega Corp.).

Western blot analysis. Whole cell lysate were collected and protein concentrations were determined using a Bradford protein Assay kit (P1511; Applygen Technologies, Inc.). Protein (40 mg) was denatured and fractionated using 12% SDS-PAGE and then electrophoretically transferred to polyvinylidene fluoride membranes (Pall Corporation, Port Washington, NY, USA). Membranes were blocked using Tris-buffered saline with Tween-20 (TBST) of 5% non-fat milk (Applygen Technologies, Inc. Applygen Technologies, Inc.) for 1 h. The membranes were subsequently probed with primary antibodies for I κ B, p-I κ B, MMP-9, COX-2, VEGF, cyclin D, c-myc, Bcl-2 and β -actin. The blots were then probed with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.). Blots were developed using a Supersignal Chemiluminescent Detection kit (Pierce Biotechnology, Inc., Rockford, IL, USA) and protein bands were visualized using a Kodak Image Station 400 and Kodak 1D 3.6 software (Kodak). β -actin was used as an internal control.

Statistical analysis. Unless otherwise stated, values are expressed as the mean \pm standard deviation. For comparisons between groups, a one-way analysis of variance was used. The significance of the difference between the means of two variables was determined using a paired Student's t-test. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. $P < 0.05$ was considered to indicate a difference, and $P < 0.01$ was considered to indicate a statistically significant difference.

Results

Ginsenoside Rg3 radiosensitizes NSCLC cells in vitro. To examine the radiosensitizing effects of Rg3 on NSCLC cells *in vitro*, a clonogenic survival assay was conducted using A549 and H1299 cells. Prior to irradiation the cells were pretreated with vehicle or ginsenoside Rg3. At the end of the experiment the colonies were counted, and survival curves were generated for the vehicle-treated and ginsenoside Rg3-treated cell groups (Fig. 1). For the A549 cell

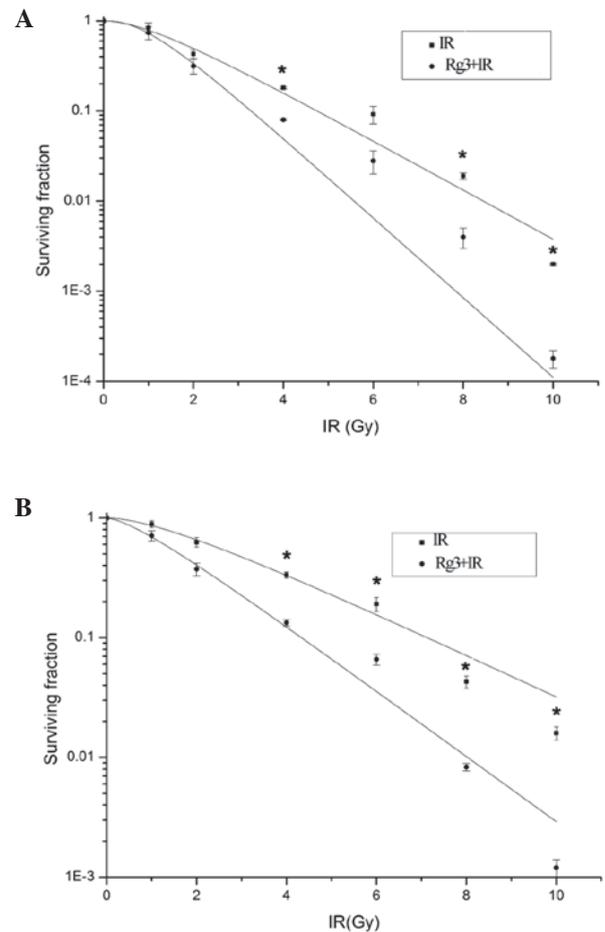


Figure 1. Dose-survival curves of (A) A549 and (B) H1299 human lung carcinoma cells following irradiation, with or without ginsenoside Rg3 treatment 24 h prior to irradiation. In the IR groups (■), the cells were exposed to various doses of radiation. In Rg3+IR groups (●), the cells were pretreated with Rg3 (25 μM). The results are expressed as the mean \pm standard deviation. * $P < 0.01$ vs. corresponding Rg3+IR group. IR, irradiation group; Rg3+IR, combination group.

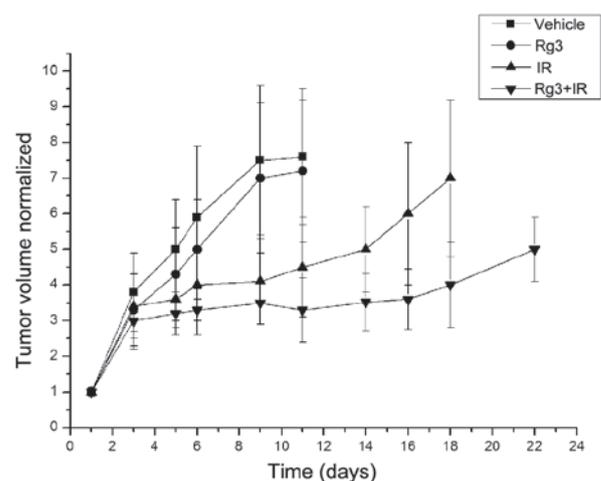


Figure 2. Ginsenoside Rg3 sensitizes tumors to radiation in C57BL/6 mice inoculated with Lewis lung carcinoma cells (LLC). Tumor volume of mice treated with control, Rg3, IR and a combination of Rg3+IR. Rg3 was administered following inoculation of LLC cells, when the tumor sizes were palpable ($\sim 200 \text{ mm}^3$, ~ 6 day post inoculation). Rg3 was administered orally twice per week, and IR was given once or combined with Rg3. Tumors were measured daily using Vernier calipers and calculated. IR, irradiation. Data are presented as the mean \pm standard deviation.

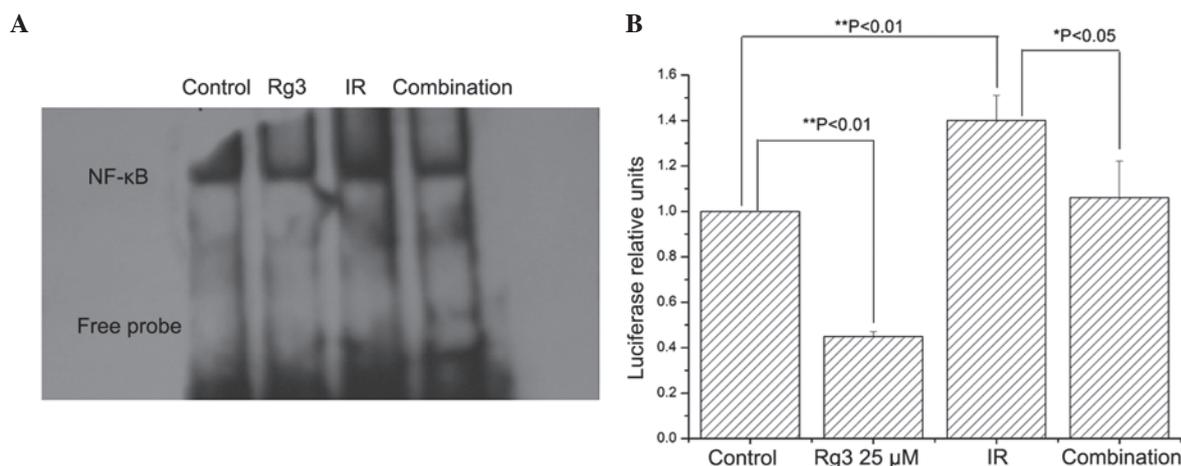


Figure 3. Ginsenoside Rg3 inhibits DNA binding and reporter gene expression in response to IR. (A) Rg3 inhibited DNA binding in response to IR. A549 human lung carcinoma cells were exposed to IR and harvested after 6 h. Nuclear extracts were probed for NF- κ B activation by electrophoretic mobility shift assay. (B) Rg3 inhibited reporter gene expression in response to IR. A549 cells stably transfected with the NF- κ B luciferase reporter plasmid, were treated with IR (8 Gy) with or without Rg3 pretreatment (25 μ M) for 24 h. Six hours after IR, the relative NF- κ B activity was measured by luciferase assay. In the Rg3 group, the cells were treated with Rg3 (25 μ M) for 24 h. The results are presented as relative activity compared with untreated control cells, and are presented as the mean \pm standard deviation from three assay wells. The experiment was repeated three times. *P<0.05 vs. the IR group; **P<0.01 vs. the control or IR group. NF- κ B, nuclear factor kappa B; IR, irradiation.

line (Fig. 1A), the radiobiological parameters of ginsenoside Rg3-treated cells were: $D_0=0.971$, $D_q=1.021$ and $N=2.752$; whereas the parameters of the vehicle-treated cells were $D_0=1.612$, $D_q=1.135$ and $N=2.124$. The SER for the A549 cells was $SER=D_0(\text{vehicle})/D_0(\text{Rg3})=1.671$. For the H1299 cell line, (Fig. 1B) the radiobiological parameters of ginsenoside Rg3-treated cells were: $D_0=1.583$, $D_q=0.674$ and $N=1.538$, whereas the parameters of the vehicle-treated cells were $D_0=2.598$, $D_q=1.463$ and $N=1.793$. The SER for the H1229 cells was $SER=D_0(\text{vehicle})/D_0(\text{Rg3})=1.639$. These results indicated that ginsenoside Rg3 may possess significant radiosensitizing effects in A549 and H1299 cells.

Ginsenoside Rg3 radiosensitizes NSCLC in vivo. The radiosensitizing effects of ginsenoside Rg3 on NSCLC were also determined *in vivo*, using an LLC xenograft model in C57BL/6 mice. Based on tumor volume measurements calculated following tumor cell implantation, the mice were randomized into four groups ($n=8$), as described in the Materials and methods. Treatment with ginsenoside Rg3 was initiated after randomization. In the untreated vehicle group, the time for the normalized tumor volume to reach five times the original volume was five days; for the ginsenoside Rg3-treated group the time was six days; for the irradiation-treated group the time was 14 days; and for the combined ginsenoside Rg3/irradiation-treated group the time was 22 days (Fig. 2). The enhancement factor was calculated as 1.78, as determined by dividing the normalized tumor growth delay of the combined groups (17 days) by the absolute tumor growth delay of the radiation-only group (nine days).

Ginsenoside Rg3 suppresses radiation-induced NF- κ B activation. Radiation induces cell death through DNA damage. In order to prevent these injuries, numerous signaling pathways in tumor cells are stimulated, among which the NF- κ B pathway has been shown to have a key role (12). Furthermore, NF- κ B has previously been identified

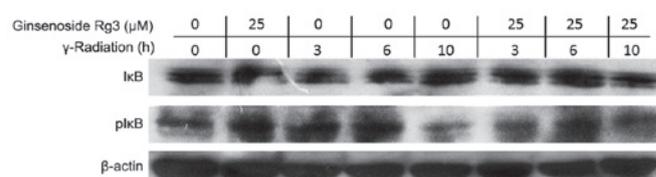


Figure 4. Ginsenoside Rg3 inhibits radiation-induced activation of proteins upstream of NF- κ B. IR induced I κ Ba degradation and phosphorylation, which was inhibited by Rg3. A549 human lung carcinoma cells were exposed to 6 Gy radiation and incubated for the indicated times. Cytoplasmic extracts were prepared and subjected to western blotting using anti-I κ Ba and anti-pI κ Ba antibodies. NF- κ B, nuclear factor kappa B; IR, irradiation; I κ Ba, inhibitor of NF- κ B.

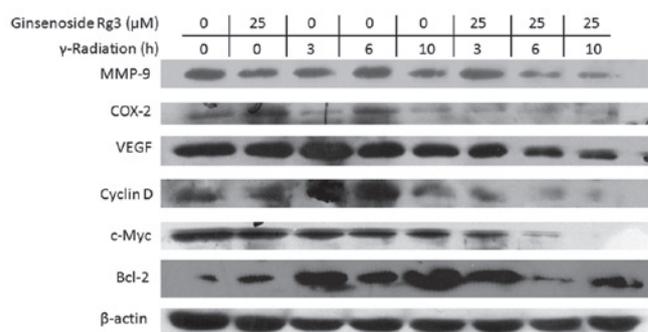


Figure 5. Ginsenoside Rg3 downregulates the protein expression levels of NF- κ B-regulated gene products in A549 human lung carcinoma cells. Western blotting demonstrated that a combination of Rg3 and radiation inhibited the expression levels of NF- κ B-dependent gene products, including B-cell lymphoma 2 (Bcl-2), cyclin D1, c-myc, cyclooxygenase (COX)-2, vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP)-9.

as a direct target of ginsenoside Rg3 (6). Therefore, the present study investigated the effects of ginsenoside Rg3 on radiation-induced NF- κ B activation. Irradiation increased NF- κ B DNA binding activity, which was suppressed by

pretreatment with ginsenoside Rg3 (Fig. 3A). These results were confirmed by NF- κ B luciferase reporter assays (Fig. 3B). These data suggested that ginsenoside Rg3 may suppress radiation-induced NF- κ B activation in NSCLC cells.

Ginsenoside Rg3 inhibits radiation-induced I κ B phosphorylation and degradation. In order to investigate how ginsenoside Rg3 was able to suppress radiation-induced NF- κ B activation, the phosphorylation status of I κ B was determined by western blot analysis. I κ B phosphorylation leads to degradation of I κ B and the release of cytoplasmic NF- κ B for nuclear translocation and DNA binding (7). Irradiation decreased the protein expression levels of I κ B, which was most obvious 3 h after radiation, whereas treatment with ginsenoside Rg3 (25 μ M) significantly inhibited this radiation-induced decrease in I κ B protein expression levels (Fig. 4).

Ginsenoside Rg3 downregulates the protein expression levels of NF- κ B-regulated genes. NF- κ B regulates tumor radioresistance through regulating the expression of Bcl-2, cyclin D1, c-myc, COX-2, VEGF and MMP-9 (13). Therefore, the present study examined the effects of radiation and ginsenoside Rg3 on the protein expression levels of these gene products by western blot analysis in the A549 cell line (Fig. 5). Treatment with ginsenoside Rg3 downregulated radiation-induced expression of Bcl-2, cyclin D1, c-myc, COX-2, VEGF and MMP-9. These results suggested that ginsenoside Rg3 may sensitize NSCLC cells to radiation through modulation of NF- κ B-regulated gene products.

Discussion

Radiotherapy has an important role in NSCLC therapy; however, radioresistance and toxicity are barriers to its successful application. Radiosensitizers are designed to enhance the destruction of tumor cells, whilst exhibiting reduced adverse effects on normal tissues, which may partly solve the radioresistance problem. Research regarding radiosensitizers has focused on proteins associated with cell signaling and growth receptors (14). As a key transcription factor, NF- κ B regulates numerous genes participating in cell proliferation, invasion, angiogenesis, metastasis, suppression of apoptosis and treatment resistance in tumors (13). Numerous inhibitors of NF- κ B have been shown to possess radiosensitizing effects (15-19). Therefore, targeting the NF- κ B pathway may provide a novel method for improving current radiosensitizing effects in NSCLC.

Numerous studies have investigated radiosensitizer compounds; however, several of these compounds have been shown to be too toxic (20). Due to their immune-regulating effects, various Asian herbs have been reported to exhibit radiosensitizing effects *in vivo* as well as *in vitro* (21-23). Ginseng is widely used in numerous cultures, particularly in China, for the prevention and treatment of numerous types of disease, including cancer (24). Ginsenoside Rg3 is a major ginseng saponin derived from heat-processed ginseng (Sun Ginseng), which has been reported to possess anti-inflammatory and anti-tumor promoting effects via inhibiting NF- κ B activation (6). Due to the key role of NF- κ B in radio-

resistance, the present study investigated the radiosensitizing effects of ginsenoside Rg3.

The clonogenic assay is a common method used to evaluate the effects of radiation on cell death. To study the radiosensitizing effects of ginsenoside Rg3 on NSCLC, two lung carcinoma cell lines, A549 and H1299, were selected. The SER in the two cell lines was shown to be >1, which suggested that ginsenoside Rg3 exhibits radiosensitizing effects on these cells. Furthermore, the radiosensitizing effects of ginsenoside Rg3 were observed in C57BL/6 mice bearing LLC xenografts. These results indicated that ginsenoside Rg3 has the tendency to increase the curative effects of radiation therapy without obvious toxic effects, thus suggesting that ginsenoside Rg3 may possess vast potential as a novel radiosensitizer.

To study the mechanisms underlying the radiosensitizing effects of ginsenoside Rg3, EMSA and luciferase reporter assays were performed. The radiosensitizing effects of ginsenoside Rg3 were most obvious at 8 Gy (data not shown); therefore, this dosage level was selected for the subsequent studies. The EMSA demonstrated that NF- κ B DNA binding activity was markedly increased following 8 Gy irradiation, and this effect was inhibited by pretreatment with ginsenoside Rg3. The luciferase reporter assay also demonstrated that treatment with ginsenoside Rg3 inhibited radiation-induced NF- κ B expression.

NF- κ B is a ubiquitous eukaryotic transcription factor and is a dimer of Rel family proteins. NF- κ B is inhibited by I κ B and is usually sequestered in the cytoplasm, where it loses its DNA binding effect. However, when cells are exposed to extracellular stimulation, I κ B is degraded, following phosphorylation, and NF- κ B can subsequently translocate into the nucleus, where it triggers the transcription of a wide array of genes that are crucial for diverse physiological responses (13). Numerous distinct NF- κ B activation pathways have been described (25). Among them, the classical pathway has been the most-well studied. Following exposure to irradiation, I κ B kinase β , which is necessary and sufficient for phosphorylation of I κ B, is activated. I κ B is then degraded following phosphorylation, resulting in the release of NF- κ B protein. NF- κ B may then translocate into the nucleus, where it binds DNA sites, and various genes responsible for irradiation resistance are stimulated (26). The results of the present study demonstrated that by inhibiting phosphorylation of I κ B, ginsenoside Rg3 inhibited the radiation-induced activation of NF- κ B, resulting in a radiosensitizing effect. However, there may be other potential targets for the radiosensitizing effects of ginsenoside Rg3, including its anti-angiogenic effect (27).

In conclusion, the present study demonstrated that ginsenoside Rg3 exhibited a radiosensitizing effect in NSCLC cell lines and a xenograft model. Furthermore, the clinical verification of ginsenoside Rg3 as a radiosensitizer of NSCLC is of considerable interest, due to its affordability, immune regulating effect, ease of oral administration and lack of toxicity. All of these properties make ginsenoside Rg3 a promising novel anti-tumor agent for the treatment of NSCLC.

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