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ORIGINAL PAPER

Gamma irradiation-induced liver injury and its amelioration by red ginseng extract

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Abstract Radiation therapy is associated with liver damage and late liver injury. The hepatoprotective effect of Korean Red Ginseng (KRG) was determined in whole-body gamma-irradiated (γIR) mice. KRG at a dose of 10 and 50 mg/kg body weight was administrated to male C57BL/6 mice (each group, n = 5) intraperitoneally for five days before whole-body γIR (6.5 Gy). Three days after γIR, serum and liver tissue were col lected and analysed. Pretreatment with KRG suppressed serum alkaline phosphatase (ALP), alkaline aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyl-transferase (GGT) activities. It also caused a marked increase in cyclooxygenase-2 (COX-2) and tumour growth factor-β1 (TGF-β1) expression associated nuclear factor-κB (NF-κB) activation in the liver. Extracellular signal-regulated kinases (ERKs) were also activated by irradiation. KRG treatment before irradiation could strongly suppress COX-2, TGF-β1, and ERK activation in the liver. Pretreatment with KRG may alleviate the severity of radiation-induced liver injury and fibrosis.

Keywords: Korean Red Ginseng, Gamma-irradiation, Liver fibrosis, Cyclooxygenase-2, Tumour growth factor

Introduction

Radiation therapy (RT), surgery, and chemotherapy are the primary treatment modalities for various cancers[1]. RT represents a non-invasive, targeted, and potentially organ-preserving therapy because of its efficiency, availability, and specificity[2]. However, the liver can be unintentionally irradiated during whole-abdomen or whole-body RT and other procedures[3]. The liver is a highly radiosensitive organ with a threshold dose between 20 and 30 Gy[4], although hepatic nonparenchymal cells, such as sinusoidal endothelial cells, hepatic stellate cells, and Kupffer cells, are more radio-resistant than other types of cells[5]. After being irradiated, these cells release fibrosis- and cirrhosis-promoting factors that trigger a progressive cascade of hepatic lobule reconstruction and distort liver function following treatment.

Radiation-induced liver disease (RILD) is caused by acute and chronic side effects of RT[6,7]. Tissue fibrosis, necrosis, atrophy, and vascular injury can occur even months to years after completion of RT[8,9]. Late radiation-induced hepatic fibrosis is becoming an increasingly serious problem in patients receiving radiation therapy[10]. Thus, studying the pathophysiological mechanisms of RILD and development of new approaches

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for preventing or mitigating the chronic state of RILD are urgently required to improve the long-term survival and the quality of life of patients[6]. Numerous drugs for preventing and treating off-target tissue injury have been developed, including sulphhydryl compounds, antioxidants, angiotensin-converting enzyme inhibitors, and others[11]. Most of compounds have failed in clinical applications due to acute toxicity and reduced specificity for normal cells. In contrast, various phytochemicals from plants including phenolic compounds, flavonoids, and alkaloids, having numerous biological functions in addition to their antioxidant capacities, have been extensively investigated to develop safe and valuable radioprotectants[12,13].

Ginseng (the roots of *Panax ginseng* C.A. Meyer) in Korea is classified into five categories, including fresh, white, and red ginseng. White ginseng is obtained by drying fresh ginseng, and red ginseng is obtained by steaming fresh 6-year-old ginseng followed by a drying process[14]. Korean Red Ginseng (KRG) has been clinically used in East Asia for treating an atherosclerosis, liver dysfunction, vascular diseases, hypertension, and post-menopausal disorders[14,15]. KRG is generally accepted as safe for human use[16]. KRG possesses photoprotective effects against ultraviolet B irradiation[17], radiation-mediated oral mucositis[18], splenic inflammation[19], and bone loss[20]. However, the hepatoprotective effect of KRG against therapeutic gamma-irradiation has not been established.

In this study, the hepatoprotective potential of KRG supplementation was investigated in mice that received whole-body gamma-irradiation (γIR). The extent of liver damage following γIR and the hepatoprotective effect of KRG were determined by changes in serum alkaline phosphatase (ALP), alkaline aspartate aminotransferase (AST/GOT), alanine aminotransferase (ALT/GPT), and γ-glutamyl-transferase (GGT) activities. The effect of KRG on cyclooxygenase-2 (COX-2) and tumour growth factor-β1 (TGF-β1) expression, which play a critical role in hepatic fibrosis[21-23], were determined in the liver of γIR mice by immunoblot analysis. In addition, the effects of KRG on γIR-mediated changes in nuclear factor-κB (NF-κB), mitogen activated protein kinases (MAPKs), including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK), p38 MAPK, and nuclear factor E2-related factor 2 (Nrf2), were also determined by immunoblot analysis.

Results

Effects of γIR on the protein expression level of COX-2 and TGF-β1, and liver damage

Hepatic fibrotic changes are strongly associated with upregulation of COX-2 and TGF-β1 expression[7,23,24]. The expression of COX-2 and TGF-β1 in the liver after γIR (0 to 6.5 Gy) increased in a dose-dependent manner, and their expression was maximal at 6.5 Gy (Supplement Figure 1). In subsequent experiments, mice received whole-body γIR at 6.5 Gy. KRG was administered intraperitoneally for five days prior to exposure to 6.5 Gy of γIR. The extent of liver damage was determined based on serum levels of ALP, AST, ALT, and GGT with or without KRG pretreatment. As shown in Figure 1, γIR significantly increased serum levels of ALP, AST, ALT, and γ-GTP by approximately 1.95-, 1.72-, 1.59-, and 1.77-fold, respectively, compared with those in unirradiated control group. The increased activities of liver enzymes indicate that whole-body γIR at a dose of 6.5 Gy induced hepatic damage. KRG pretreatment at the dose of 10 mg/kg and 50 mg/kg significantly decreased serum levels of ALP, AST, and ALT (Figure 1) compared to those of the irradiated control group, but was less effective in decreasing the serum level of γ-GTP. These results suggest that impairment of liver function caused by whole-body γIR could be attenuated by pretreatment with KRG.

Effects of KRG on the γIR-induced protein expression levels of COX-2 and TGF-β1

The effects of KRG on the expression COX-2 and TGF-β1 were determined by immunoblot. The expression of COX-2 and TGF-β1 was significantly suppressed in KRG-treated mice liver compared to that in the irradiated control group, but the effect was not dose dependent (Figure 2). These data suggest that in addition to directly protective effects of KRG on γIR-mediated liver damage, KRG may be an inhibitor the γIR-induced increase in COX-2 and TGF-β1 protein.

Effect of KRG on the activation of ERK, JNK, and p38 MAPK in the liver of of γIR-exposed mice

Multiple intracellular signalling pathways involved in cell death and inflammatory responses can be activated by irradiation[25]. One pathway involves proteins of the mitogen-activated protein kinase (MAPK) superfamily such as ERK, JNK and p38 MAPK[26]. In addition, it has been suggested that up-regulation of COX-2 requires activation of MAPKs and related transcription factors including NF-κB as reported for carbon tetrachloride-induced liver injury in rats[27]. Therefore, the effect of KRG on the activation of ERK, JNK and p38 MAPK were determined by immunoblot in liver from γIR mice (Figure 3). Both ERK and JNK were activated, but p38 MAPK was not in livers from γIR mice. ERK was phosphorylated to a greater extent than JNK. Pretreatment of KRG prior to γIR significantly atten-

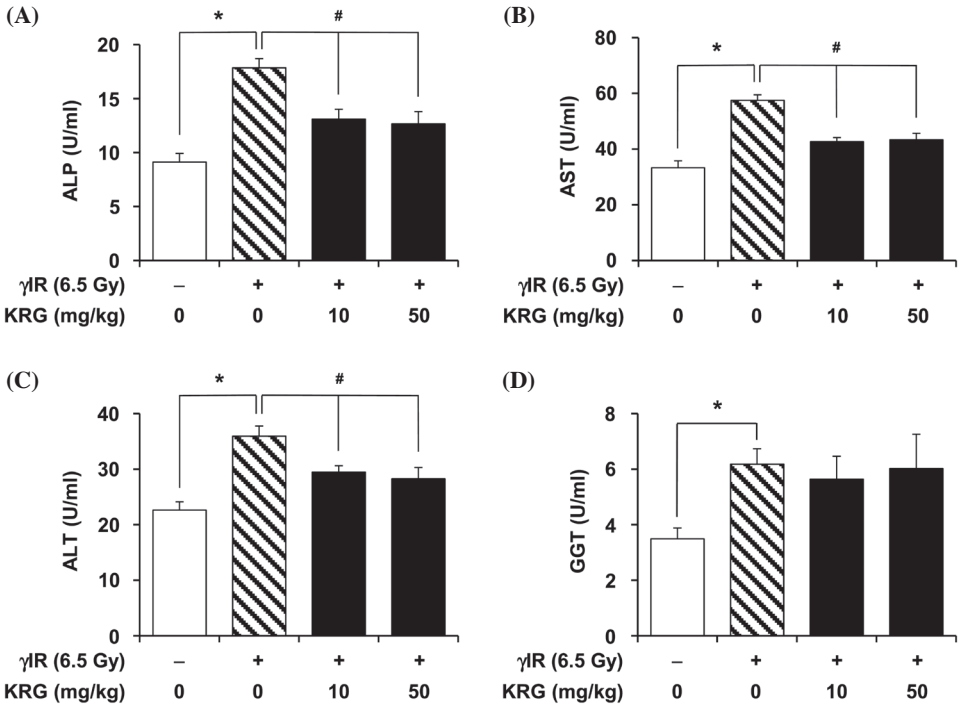

Figure 1. Effect of KRG on liver function. Groups of mice (n = 5) received no or whole-body γIR (6.5 Gy) after pretreatment for 5 days with 0, 10, or 50 mg/kg KRG. Three days later, serum levels of ALP (A), AST (B), ALT (C), and GGT (D) were determined by colorimetry method. Significant differences are represented by an asterisk (*), where $P < 0.05$ compared to non-irradiated control, and a hashmark (#), where $P < 0.05$ compared to γIR-irradiation alone. γIR; whole-body gamma irradiation, KRG; the extract of Korean Red Ginseng.

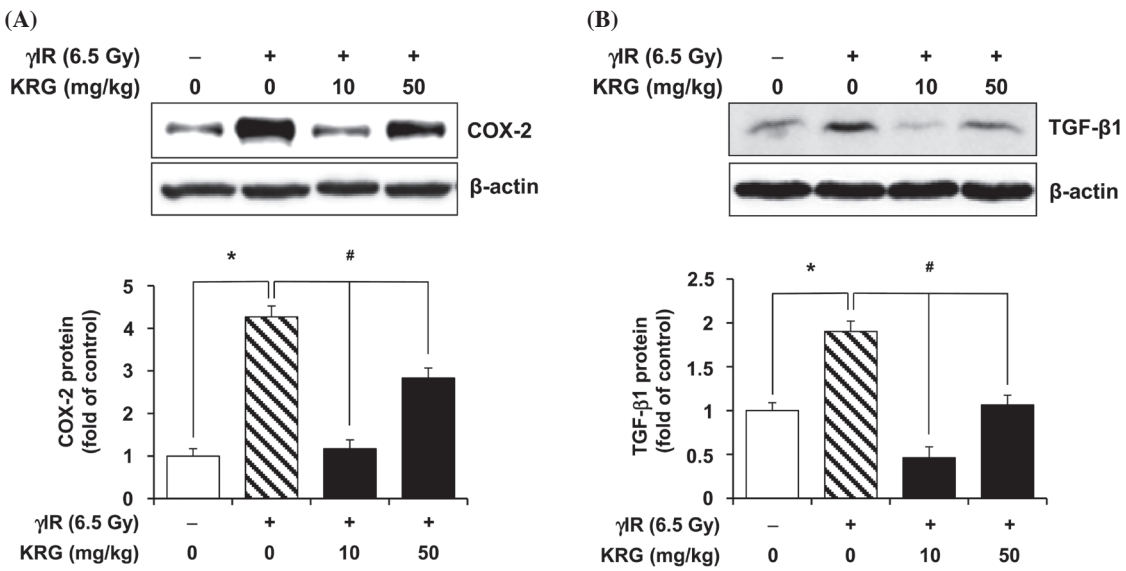

Figure 2. Effect of KRG on the expression of COX-2 and TGF-β1 in γIR-exposed murine liver. Mice received whole-body γIR with 6.5 Gy in the presence or absence of KRG pretreatment for 5 days (each group, n = 5). The expression of COX-2 (A) and TGF-β1 (B) in murine liver was determined by immunoblot. Values are obtained from densitometric analysis of immunoblot and expressed as a mean ± SEM. Significant differences are represented by an asterisk (*), where $P < 0.05$ compared to non-irradiated control, and a hashmark (#), where $P < 0.05$ compared to γIR-irradiation alone). γIR; whole-body gamma irradiation, KRG; the extract of Korean Red Ginseng.

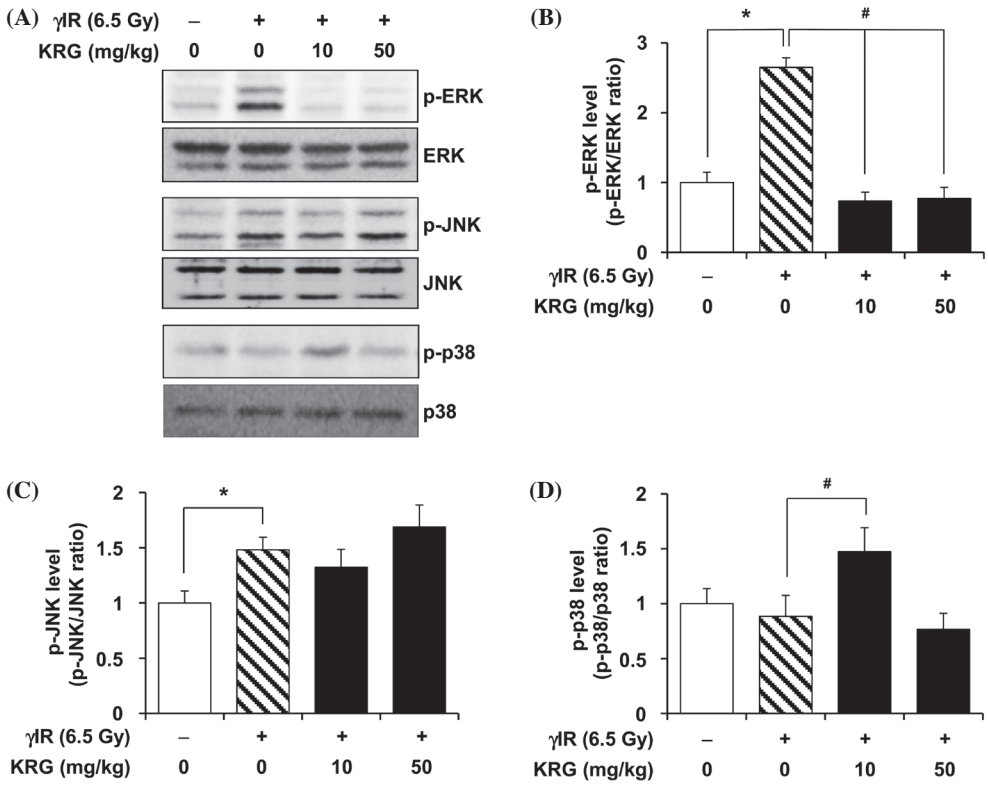

Figure 3. Effect of KRG on activation of ERK, JNK, and p38 MAPK in γIR-exposed murine liver. Mice received γIR with 6.5 Gy in the presence or absence of KRG pretreatment for 5 days (each group, n = 5). The phosphorylation of ERK, JNK, and p38 MAPK was determined by immunoblot. (A) The representative image of immunoblot, (B) ERK, (C) JNK, and (D) p38 MAPK. Values are obtained from densitometric analysis of immunoblot and expressed as mean ± SEM. Significant differences are represented by an asterisk (*), where $P < 0.05$ compared to non-irradiated control, a hashmark (#), where $P < 0.05$ compared to γIR-irradiation alone. γIR; whole-body gamma irradiation, MAPK; mitogen activated protein kinase, KRG; the extract of Korean Red Ginseng.

uated the activation of ERK as compared to that in the livers of the irradiated control group, whereas the suppression of JNK activation by KRG supplementation was not significant. p38 MAPK was not activated but rather suppressed by γIR. The highest level of p38 MAPK activation was seen in mice supplemented with 10 mg/kg/day KRG.

Effect of KRG on the activation of NF-κB in the liver of γIR-exposed mice

The expression of COX-2 and/or TGF-β1 can be driven by the activation of NF-κB[28]. To determine whether the suppression of COX-2 expression via KRG supplementation is associated with the inhibition of NF-κB, the changes in the protein level of phosphorylated IκB (p-IκB) was determined by immunoblot. As depicted in Figure 4A, γIR led to an increase of p-IκB which indicates the activation of NF-κB. The γIR-mediated increase of p-IκB was suppressed by supplementation with KRG at 10 mg/kg but less extent by KRG at 50 mg/kg.

Effect of inhibition of NF-κB and ERK on the expression of TGF-β1 in liver cells

To determine the involvement of ERK and NF-κB on γIR-induced TGF-β1 expression, liver cells were treated with 2.5 Gy in the presence or absence of 50 nM Withaferin A (an NF-κB inhibitor) and 10 μM PD98059 (an ERK inhibitor). The expression of TGF-β1 was significantly suppressed in the presence of NF-κB and ERK inhibitors (Figure 4B). This result indirectly suggests that the suppressive effect of KRG on the expression of TGF-β1 shown in Figure 2B may act through an inhibitory effect on NF-κB and ERK signlaing.

Effect of KRG on the expression of heme oxygenase-1

Heme oxygenase-1 (HO-1) has potent antioxidant as well as anti-inflammatory functions which are tissue protective when expression is induced[29]. Following γIR, the HO-1 expression in the liver was not induced but rather suppressed compared to samples from un-

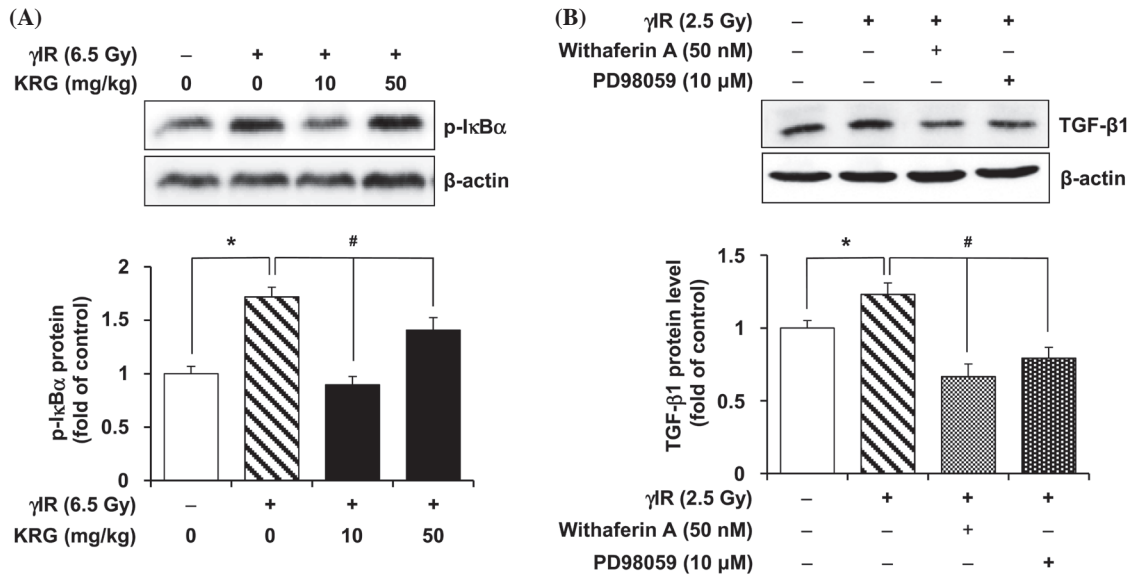

Figure 4. Effect of KRG on the γIR-induced activation NF-κB in murine liver, and the suppression of TGF-β1 expression by NF-κB and ERK inhibitors in liver cells. Groups of mice (n = 5) received γIR with 6.5 Gy after being pretreated with 0, 10, or 50 mg/kg KRG. The activation of NF-κB was determined by the phosphorylation of $I_kB\alpha$ ($p\text{-}I_kB\alpha$) using immunoblot (A). Liver cells were exposed to γIR with 2.5 Gy in the presence or absence of either withaferin A (NF-κB inhibitor, 50 nM) or PD98059 (ERK inhibitor, 10 μM) (B). Values are obtained from densitometric analysis of immunoblots and expressed as mean ± SEM. Significant differences are represented by an asterisk (*), where $P < 0.05$ compared to non-irradiated control, and a hashmark (#), where $P < 0.05$ compared to γIR-irradiation alone. γIR; whole-body gamma irradiation, KRG; the extract of Korean Red Ginseng.

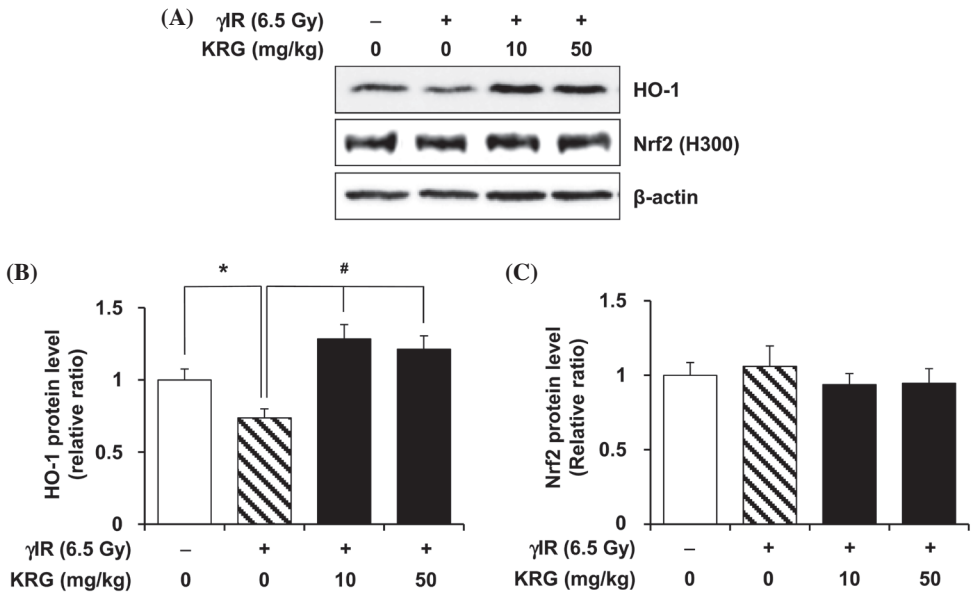

Figure 5. Effect of KRG on the expression of Nrf2 in γIR-exposed murine liver. Groups of mice (n = 5) received γIR with 6.5 Gy after pretreatment for 5 days with 0, 10, or 50 mg/kg KRG. The representative image of immunoblot (A), HO-1 (B) and Nrf2 (C). Values are obtained from densitometric analysis of immunoblot and expressed as a mean ± SEM. Significant differences are represented by an asterisk (*), where $P < 0.05$ compared to non-irradiated control, and a hashmark (#), where $P < 0.05$ compared to γIR-irradiation alone. γIR; whole-body gamma irradiation, KRG; the extract of Korean Red Ginseng.

treated control mice. Livers from the groups of mice supplemented with KRG showed a significant increase of HO-1 as compared to the level in livers from the irradiated control group (Figure 5B). Nrf2 is a redox

sensitive transcription factor and can be involved in the induction of HO-1[30]. To test the involvement of Nrf2 in KRG-mediated hepatoprotective effect against γIR, the change in the protein level of Nrf2 was determined by immunoblot. There was no change in the expression of Nrf2 in samples from the treated groups of mice as compared to samples from unirradiated control mice (Figure 5C). This result may exclude the involvement of Nrf2 pathway in the hepatoprotective effect of KRG in γIR insults.

Discussion

In this study, supplementation with KRG before γIR could protect liver damage. The inhibitory effect of KRG on the activation of NF-κB and ERK, which play a role in the upregulation of COX-2 and TGF-β1 expression, will be beneficial in suppressing hepatic fibrinogenesis followed by γIR exposure.

The liver performs vital functions including; bile production, metabolism of ingested nutrients, and detoxification. However, it is highly vulnerable to damage from γIR exposure[3,7,31]. Radiation-induced toxicity in normal liver tissue is associated with the development of multiple side effects that may lead to chronic complications including pathogenic fibrosis that will progress to cirrhosis and portal vein hypertension, or hepatocellular carcinoma. In searching for a suitable radioprotectant for the liver, we found that the supplement KRG might serve as an alternative composition for attenuating hepatic damage and suppressing pro-fibrotic signals such as COX-2 (Figure 2A), TGF-β1 (Figure 2B) and NF-κB (Figure 4A). Liver functions were markedly compromised by γIR as evidenced by the resultant increase in serum levels of ALP, AST, ALT and GGT. The impairment in liver function was markedly attenuated by pretreatment of mice with KRG (Figure 1). The induction of HO-1 is viewed as contributing a protective role against γIR-induced liver injury[29]. In our experiment, the expression of HO-1 decreased in the livers of γIR-exposed mice. However, KRG pretreatment alleviated γIR-induced liver damage (Figure 1) and allowed high level HO-1 induction following γIR exposure (Figure 5B). The restoration of HO-1 induction by KRG pretreatment could be, at least in part, involved in the hepatoprotective mechanism of KRG in the liver. However, the exact role of HO-1 in γIR-exposed liver needs to be further investigated.

Radiation injury results in the up-regulation of COX-2 and a concomitant increase in prostaglandin E2 (PGE2) synthesis in mice[32]. Although there is controversy about the precise role of COX-2 in the liver[33,34], the upregulation of COX-2 may promote the activation and proliferation of hepatic stellate cells, leading to liver fibrosis[34]. The expression of COX-2 and TGF-β1 in the liver was significantly up-regulated by γIR insult in mice (Figure 2). There was no change in the protein level of Nrf2 (Figure 5C), which upregulates the hepatoprotective protein, HO-1. Enhanced TGF-β signalling associated with chronic liver damage leads to the differentiation of stellate cells to myofibroblasts and is accompanied by massive hepatocyte cell death, and further contributes to the promotion of liver fibrosis and ultimately cirrhosis[35]. Therefore, therapy targeting suppression of TGF-β[7], NF-κB, and COX-2 expression in the liver may mitigate subsequent liver pathology[24] and restore liver function after radiation therapy[3,7]. Based on this theory, the suppression of COX-2 and TGF-β′1 expression by KRG supplementation may contribute to suppressing hepatic fibrosis following γIR.

It has been suggested that MAPK pathways control cell functions such as apoptosis or proliferation after irradiation[26]. It is also assumed that the activation MAPK is involved in tissue-specific regulation following γIR insult. For example, p38 MAPK and JNK are prominent in γIR-exposed splenocytes and human keratinocyte HaCaT cells, respectively[36]. In the murine liver, γIR caused marked activation of ERK, rather than JNK, without activation of p38 MAPK (Figure 3). Several studies have documented that the ERK signalling pathway is involved in hepatic fibrosis[26,37]. ERK signalling further contributes to the p70 S6 kinase activation induced by growth factors, ultraviolet and ionising radiation[26]. ERK 1/2 and p70 S6 kinases are highly activated in damaged liver cells during tissue regeneration and remodelling. However, aberrant activation of these kinases is involved in late hepatic fibrosis[38]. In agreement with these results, exposure of γIR enhanced TGF-β1 expression and activation of ERK in the murine liver. While the up-regulation of pro-fibrotic proteins could be attenuated by KRG supplementation, the exact mode of control of COX-2 expression by KRG supplementation is not clear. The inhibitory effect of KRG on the NF-κB and ERK activation may be, at least in part, associated with suppression of COX-2 and TGF-β1 induction (Figure 4). It should be mentioned that the underlying radioprotective mechanism of KRG shown in the liver could also be linked, either directly or indirectly, to antioxidant mechanisms or free radical scavenging[39]. Finally, while this study shows the hepatoprotective effect of KRG supplementation against γIR insult in the liver, whether KRG augments or reduces the efficacy of radiation therapy in cancer treatment has not been tested. The identification of the active compound(s) in KRG contributing to suppression of hepatic fibrotic progression will also be important

to developing a new radioprotectant. These topics may be a subject for future research.

Taken together, KRG supplementation could ameliorate γIR-mediated liver dysfunction. In addition, the γIR activation of pro-fibrotic proteins such as COX-2, ERK, NF-κB, and TGF-β1 was markedly suppressed after KRG supplementation. Therefore, the observed anti-fibrotic potential of KRG may be beneficial for alleviating the radiation-induced liver disease.

Materials & Methods

Chemicals and Reagents

Withaferin A (a NF-κB inhibitor) and PD98059 (an ERK inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against COX-2 and TGF-β1 were purchased from BD Transduction Laboratories (Lexington, KY). Anti Nrf2 (H-300) antibody was purchased from Santa Cruz (Dallas, TX). Other antibodies, including ERK1/2, p38 MAPK, JNK, phosphor-ERK1/2, phosphor-p38, phosphor-JNK, and Nrf2, were obtained from Cell Signaling Technology (Beverley, MA). The RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY).

Experimental animals

Six-week-old male C57BL/6 mice were obtained from Jung-Ang Laboratory Animal Company (Daejeon, South Korea) and housed with access to food and water ad libitum. Mice were maintained in a registered Experimental Animal Facility, and protocols were approved by the Animal Care Committee at the Radiation Health Research Institute (Seoul, Korea). The studies were performed in accordance with the Institutional Animal Care and Use Committee Guidelines. During the study, all animals were maintained at a temperature of 23 ± 1 °C and humidity of 55 ± 5% with 10-18 air changes/h under a 12/12 h light/dark cycle. The animals were divided randomly into four groups of five mice each: 1) normal control; 2) irradiated; 3) 10 mg/kg/day of KRG administrated and irradiated; and 4) 50 mg/kg/day of KRG administrated and irradiated.

Preparation of KRG

KRG was obtained from KGC (Korea Ginseng Corporation, Seoul, Korea). KRG contains ginsenoside Rb1 (G-Rb1): 8.27 mg/g, G-Rb2: 3.22 mg/g, G-Rc: 3.90 mg/g, G-Rd: 1.09 mg/g, G-Re: 2.58 mg/g, G-Rf: 1.61 mg/g, G-Rg1: 2.01 mg/g, G-Rg2s: 1.35 mg/g, G-Rg3s: 1.04 mg/g, and other minor ginsenosides. The extract of KRG obtained from KGC was dissolved in phosphate-buffered saline (PBS) at a final concentration of 30 mg/mL and frozen until use.

Radiation facility

The radiation equipment was a commercial Cesium-137 (^{137}Cs) source emitting gamma rays (IBL 437C, CIS Bio International S.A., Gif-sur-Yvette, France). The used irradiation equipment had been regularly examined and validated for dose accuracy by the national regulatory organisation, Korea Institute of Nuclear Safety (KINS, Daejeon).

Gamma-irradiation and collection of samples

KRG was administered to the mice intraperitoneally (i.p.) at a dose of 10 and 50 mg/kg/day for five days. Untreated control and γIR control mice received equal volume of normal saline for five days. Thereafter, the mice were exposed to γIR as previously described[19]. Briefly, the mice received whole-body irradiated from 0 to 6.493 Gy using 0.8 Gy/min to calculate exposure time. The radiation dose was validated through thermoluminescent dosimeters placed within the exposure field. Three days after γIR, blood samples were collected from the posterior vena cava of each animal, and the liver was removed under ether anaesthesia. During the experiment period, there was no mortality and no unexpected changes in general behaviour and response was observed.

Biochemical analysis

Blood serum samples were centrifuged at 3,000 × g for 10 minutes, and then serum was collected and stored at − 80°C until analysis. Enzyme activities for ALP, AST, ALT, and GGT were estimated using Enzymatic Assay Kits (Asan Pharm. Co., Korea) following the manufacturer's instructions.

Preparation of liver tissue protein from irradiated mice

The liver was placed in cold RPMI1640 medium and passed through a 70-μm nylon mesh (BD Biosciences, CA) to produce a single-cell suspension. The suspension was centrifuged at 250 × g for 5 min, and the supernatant was removed. The pellet was lysed in lysis buffer using tissue grinder, and liver tissue protein was collected by centrifugation at 15,000 × g for 30 min.

Western blotting

The protein content of liver cell was measured using a Bio-Rad protein assay kit (Hercules, CA, USA). Samples were diluted with 1 × lysis buffer contain-

ing 1% β-mercaptoethanol. Equal amounts of cellular protein (50 μg) were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with the targeted antibody and then with horseradish peroxidase-conjugated secondary antibody to IgG. Immunoreactive proteins were visualised using the ECL Western blot detection system. The protein level was compared to a loading control, such as β-actin and/or non-phosphorylated protein.

Statistical analysis

Each experiment was repeated four or five times, and data are expressed as mean ± S.E.M. Significance was determined by one-way analysis of variance (ANOVA) followed by a modified t-test with Bonferroni's correction for comparisons between individual groups using SPSS, version 12 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ indicated significance.

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Conflict of Interest Seon-A Jang declares no conflict of interest. Sung Ryul Lee declares no conflict of interest. Hyun Jung Koo declares no conflict of interest. Jin Woo Lee declares no conflict of interest. Yuna Park declares no conflict of interest. Seung Namkoong, declares no conflict of interest. Myung Kyum Kim, declares no conflict of interest. Se Chan Kang declares no conflict of interest. Eun-Hwa Sohn declares no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Human and animal rights All experimental procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23) and National Animal Welfare Law in Korea. The experimental animal facility and protocols were approved by the Institutional Animal Care and Use Committee of Radiation Health Research Institute (Seoul, Korea).

Author's contributions Seon-A Jang, Sung Ryul Lee, Se Chan Kang and Eun-Hwa Sohn designed the research study. Seon-A Jang, Hyun Jung Koo, Jin Woo Lee, Yuna Park and Seung Namkoong performed the experiments and analyzed data. Myung Kyum Kim, Se Chan Kang, Sung Ryul Lee and Eun-Hwa Sohn interpreted data and wrote manuscript.

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