J Ginseng Res 44 (2020) 843–848

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Technical Note

Ginsenoside-Rp1 inhibits radiation-induced effects in lipopolysaccharide-stimulated J774A.1 macrophages and suppresses phenotypic variation in CT26 colon cancer cells

Ji Sue Baik [1,☆], You Na Seo [1,☆], Joo Mi Yi [2], Man Hee Rhee [3], Moon-Taek Park [1,**], Sung Dae Kim [1,*]

[1] Research Center, Dongnam Institute of Radiological & Medical Sciences, Busan, Republic of Korea
[2] Department of Microbiology and Immunology, College of Medicine, Inje University, Busan, Republic of Korea
[3] Department of Veterinary Medicine, College of Veterinary Medicine, Kyungpook National University, Daegu, Republic of Korea

ARTICLE INFO

Article history:
Received 21 February 2019
Received in Revised form
6 January 2020
Accepted 21 January 2020
Available online 31 January 2020

Keywords:
Cancer
Ginsenoside-Rp1
Macrophages
Panax ginseng
Radiation

ABSTRACT

This study investigated the inhibitory effect of ginsenoside-Rp1 (G-Rp1) on the ionizing radiation (IR)-induced response in lipopolysaccharide (LPS)-stimulated macrophages and its effects on the malignancy of tumor cells. G-Rp1 inhibited the activation of IR-induced DNA damage-related signaling molecules and thereby interfered with the IR-increased production of nitric oxide (NO) and interleukin (IL)-1β. The inhibitory effect of G-Rp1 increased the survival rate of mice inoculated with CT26 colon cancer cells by suppressing the phenotypic variation of tumor cells induced by conditioned medium obtained from IR- and LPS-treated J774A.1 macrophages.

Ginsenoside-Rp1 (G-Rp1) is a ginseng saponin produced by the hydrogenation and reduction of crude ginsenosides obtained from *Panax ginseng* Meyer. Several pharmaceutical applications of G-Rp1, including treatments for arteriosclerosis [1], inflammatory disease [2], and cancer [3], have been reported. Despite its long history, none of the investigations have elucidated the role of G-Rp1 in combating ionizing radiation (IR) exposure.

Macrophages, which are key players in innate immunity and the tumor environment, are considered radioresistant. We previously reported that IR enhanced lipopolysaccharide (LPS)-induced NO synthesis and IL-1β production [4]. These two inflammatory mediators were found to be effective indicators of the pathophysiological response to IR. Therefore, we evaluated the effect of G-Rp1 on the synthesis of IR-enhanced, LPS-stimulated pro-inflammatory mediators *in vitro* and the associated clinical implications *in vivo*.

G-Rp1 (purity 99%), prepared using established protocols, was obtained from the Ambo Institute (Daejeon, Korea) [2]. G-Rp1 was dissolved in 100% dimethyl sulfoxide (DMSO) and the cells were treated with the indicated doses of G-Rp1 or 0.1% DMSO (control). The chemical structure of G-Rp1 and its high-performance liquid chromatography (HPLC) data are shown in Fig. 1B. Up to 10 μM of G-Rp1 was confirmed to be non-toxic to the cell lines used in this study through the CCK8-assay (Fig. 1C). LPS and all other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless indicated otherwise. The J774A.1 cells and CT26 colon cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). To investigate the inhibitory effect of G-Rp1 on IR-induced effects in LPS-treated macrophages, J774A.1 cells (2×10^5 cells/mL) were pretreated with or without G-Rp1 (2.5 to 10 μM) for 30 min and exposed to gamma irradiation using a Biobeam 8000 (137Cs source) cell irradiator (Gamma-Service Medical GmbH, Leipzig, Germany) at a dose rate of 2.5 Gy/min and incubated at 37°C for 24 h. The cells were exposed to LPS (0.1 μg/mL) with or without pretreatment with G-Rp1 (2.5 to 10 μM) for 30 min

* Corresponding author. Research Center, Dongnam Institute of Radiological & Medical Sciences, Busan, 46033, Republic of Korea.
** Corresponding author. Research Center, Dongnam Institute of Radiological & Medical Sciences, Busan, 46033, Republic of Korea.
E-mail addresses: mtpark@dirams.re.kr (M.-T. Park), sdkim@dirams.re.kr (S.D. Kim).
☆ Ji Sue Baik and You Na Seo contributed equally to this study.

https://doi.org/10.1016/j.jgr.2020.01.006
p1226-8453 e2093-4947/$ – see front matter

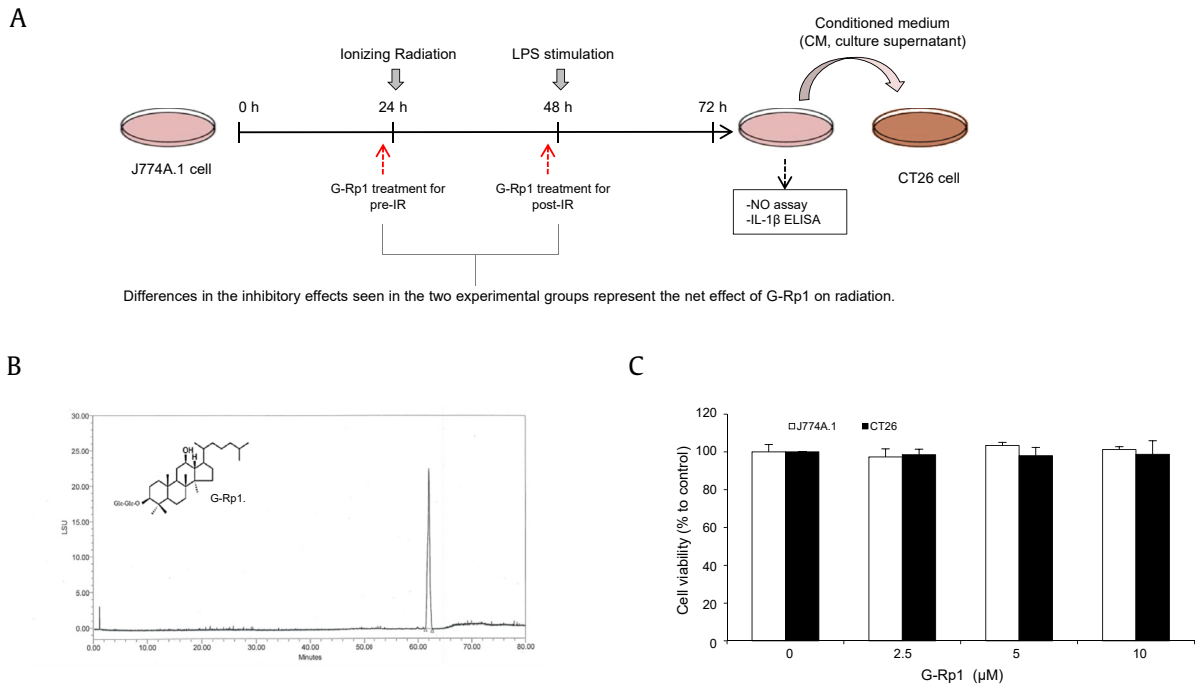

Fig. 1. Schematic diagram of the study. (A) J774A.1 cells (2×10^5 cells/mL) were irradiated (2 Gy) using a blood gamma irradiator and incubated for 24 h. The cells were treated with LPS (0.1 μg/mL) and 24 h later, the CM was used for NO_2 or IL-1β determination. In this process, the cells were treated with ginsenoside-Rp1 (G-Rp1) 30 min before irradiation exposure (pre-IR) or 30 min before LPS treatment (post-IR), as illustrated. The differences in the inhibitory effects seen in the two experimental groups represent the net inhibitory effect of G-Rp1 on radiation exposure. After that, CM (culture supernatant) obtained from J774A.1 cells treated as indicated was transferred to CT26 colon cancer cells to investigate the phenotypic variations. (B) The chemical structure of G-Rp1 with the HPLC data. (C) The effect of G-Rp1 on the cell viability in J774A.1 cells and CT26 colon cancer cells.

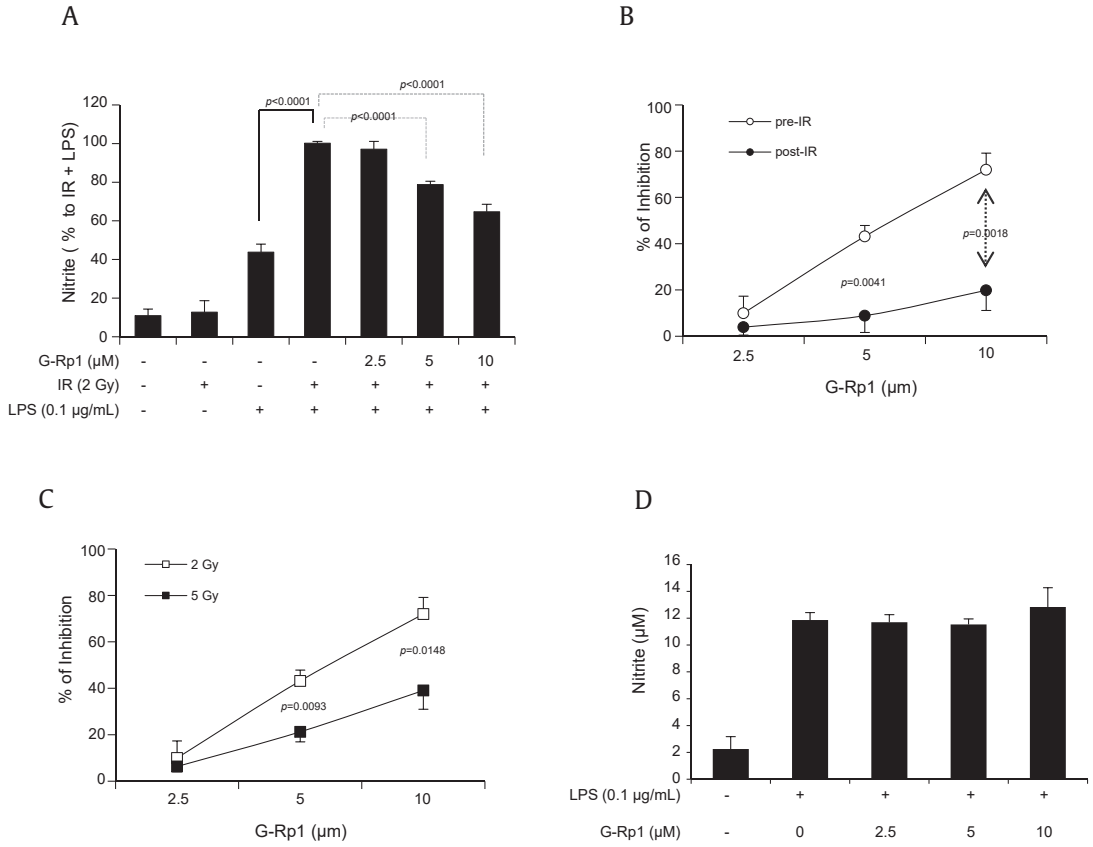

Fig. 2. G-Rp1 inhibits NO synthesis in IR-enhanced LPS-stimulated J774A.1 macrophage cells. (A) Pretreatment with G-Rp1 (2.5, 5, and 10 μM) inhibits IR-potentiated LPS-stimulated NO production from J774A.1 cells in a concentration-dependent manner. (B) The relative inhibition of NO production by G-Rp1 was compared by treating with G-Rp1 before and after IR. (C) The proportion of NO inhibition by G-Rp1 in cells irradiated with 2 Gy and 5 Gy was analyzed. (D) The effect of G-Rp1 on the LPS (0.1 μg/mL)-induced NO production in J774A.1 murine macrophage cells.

and incubated for another 24 h. The differences in the inhibitory effects seen in the two experimental groups represent the net effect of G-Rp1 on radiation (Fig. 1A). NO levels in the conditioned medium (CM) (culture supernatant) were analyzed using Griess reagent as described previously [4]. The absorbance was evaluated at 570 nm. After NO estimation in the culture medium, the IL-1β levels were quantified according to the manufacturer's procedure (R&D Systems, Minneapolis, MN, USA). All animal experimental procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Dongnam Institute of Radiological and Medical Sciences (DIRAMS) (DI-2018-017). Student *t* tests were performed to analyze statistical significance using SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA). A *p*-value of less than 0.05 was considered significant.

As shown in Fig. 2A, single IR did not significantly increase the NO levels in J774A.1 cells compared to the control cells. Meanwhile, increased NO production was observed in irradiated cells in response to LPS stimulation. In addition, G-Rp1 inhibited the IR (2 Gy)-enhanced, LPS-induced NO production in a concentration-dependent manner. We further compared the inhibitory effect of G-Rp1 treatment before and after IR exposure on the LPS-stimulated NO production. As shown in Fig. 2B, the G-Rp1 inhibition of radiation-enhanced NO production was greater when it was administered before, rather than after, the cells were irradiated. The half-maximal inhibitory concentration (IC50) of G-Rp1 on the NO production in IR-enhanced, LPS-induced J774A.1 cells was 8.1 ± 2.0 μM and >10 μM, respectively. We further analyzed the inhibitory effect of G-Rp1 by increasing the IR dose from 2 Gy to 5 Gy. As shown in Fig. 2C, an increase in radiation dose from 2 Gy to 5 Gy decreased the inhibition by G-Rp1. We also tested the effect of G-Rp1 on the LPS-induced NO production. As shown in Fig. 2D, G-Rp1 had no significant inhibitory effect on the LPS-induced NO production in J774A.1 macrophage cells. The NO synthesis results strongly suggest that G-Rp1 inhibited the effects of radiation on LPS-stimulated J774A.1 murine macrophages.

We previously reported that IR increased the IL-1β levels in LPS-stimulated macrophages [4]. Hence, we evaluated the effect of G-Rp1 on IR-mediated IL-1β synthesis in LPS-stimulated J77A.1 macrophages. As shown in Fig. 3A, radiation insult resulted in enhanced LPS-induced IL-1β release. Pretreatment with G-Rp1 strongly attenuated the IR-enhanced LPS-induced IL-1β in a concentration-dependent manner with an IC50 of 5.4 ± 0.3 μM. Moreover, G-Rp1 pretreatment showed a more potent inhibitory effect compared to post-irradiation exposure (Fig. 3B). An increase in radiation dose from 2 Gy to 5 Gy decreased the inhibition by G-Rp1 (Fig. 3C). Furthermore, G-Rp1 inhibited IR-induced caspase-1 (an IL-1-converting enzyme) without affecting the NLRP3 expression (Fig. 3D). All these findings suggest that G-Rp1 had strong protective effects against IR exposure in macrophages.

DNA damage-related genes, such as *H2AX*, *CHK1*, and *CHK2*, produce key proteins mediating the radiation-induced pathophysiological response. Ibuki et al suggested that radiation-induced DNA damage is a key molecular mechanism underlying the enhanced NO production in macrophage cells exposed to radiation [5]. Therefore, we assessed the effect of G-Rp1 (10 μM) on gene activation following IR-induced DNA damage. Western blot analysis showed that G-Rp1 significantly inhibited the IR-mediated H2AX, chk1, and chk2 expression (Fig. 4A). Using confocal microscopy, we also demonstrated that G-Rp1 inhibited IR-induced H2AX expression, an upstream target of IR, rather than chk1 and chk2 (Fig. 4B). To identify the upstream molecular target of G-Rp1, we used an *in vitro* kinase assay against ATM and ATR. G-Rp1 had no direct

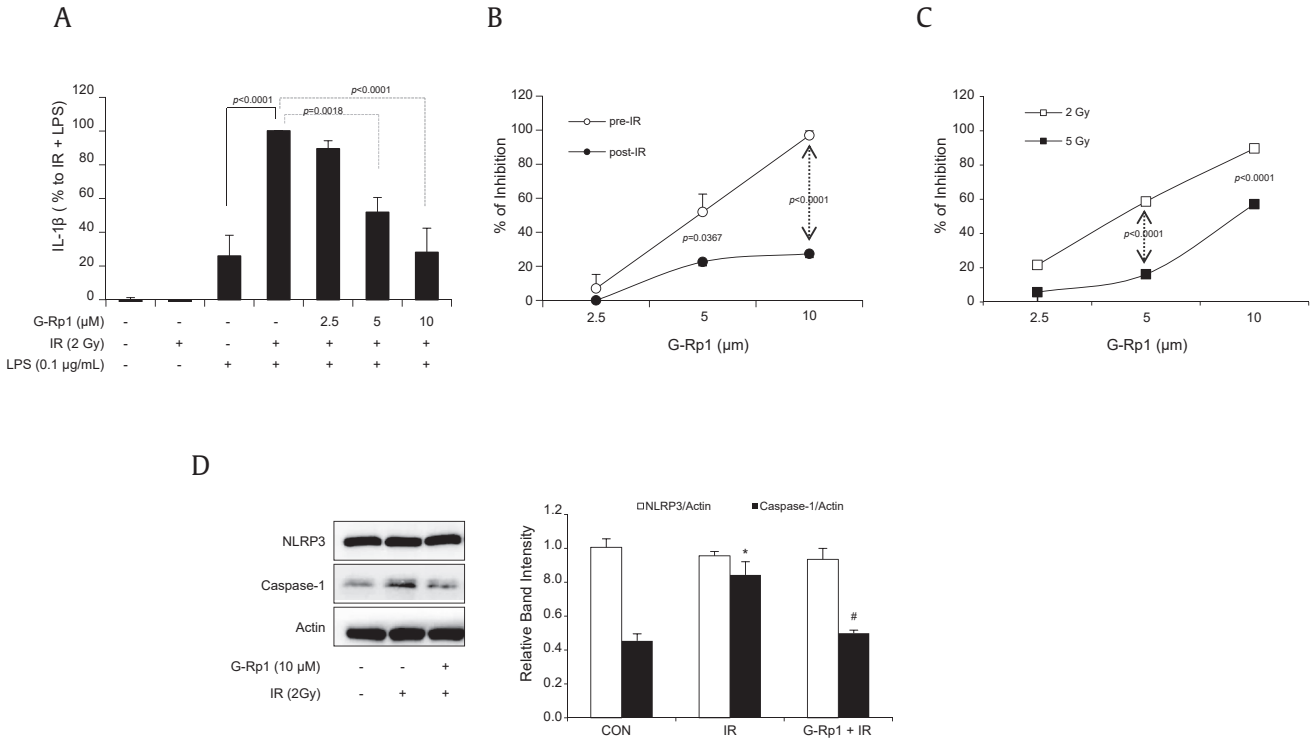

Fig. 3. G-Rp1 inhibits IL-1β production in IR-enhanced LPS-stimulated J774A.1 cells. (A) Pretreatment with G-Rp1 (2.5, 5, and 10 μM) inhibits IR-potentiated LPS-stimulated soluble IL-1β secretion from J774A.1 cells in a concentration-dependent manner. (B) The relative inhibition of IL-1β production by G-Rp1 was compared to treatment with G-Rp1 before and after IR. (C) The inhibition of IL-1β by G-Rp1 was analyzed in cells exposed to 2 Gy and 5 Gy irradiation and expressed as a percentage. (D) J774A.1 cells (2×10^5 cells/mL) were incubated with and without G-Rp1 (10 μM) for 30 min and irradiated (2 Gy) using a blood gamma irradiator, followed by incubation at 37°C for 24 h. The NLRP3 and caspase-1 expression levels were analyzed by Western blots. *$p < 0.05$ versus LPS. #$p < 0.05$ versus IR-treated group.

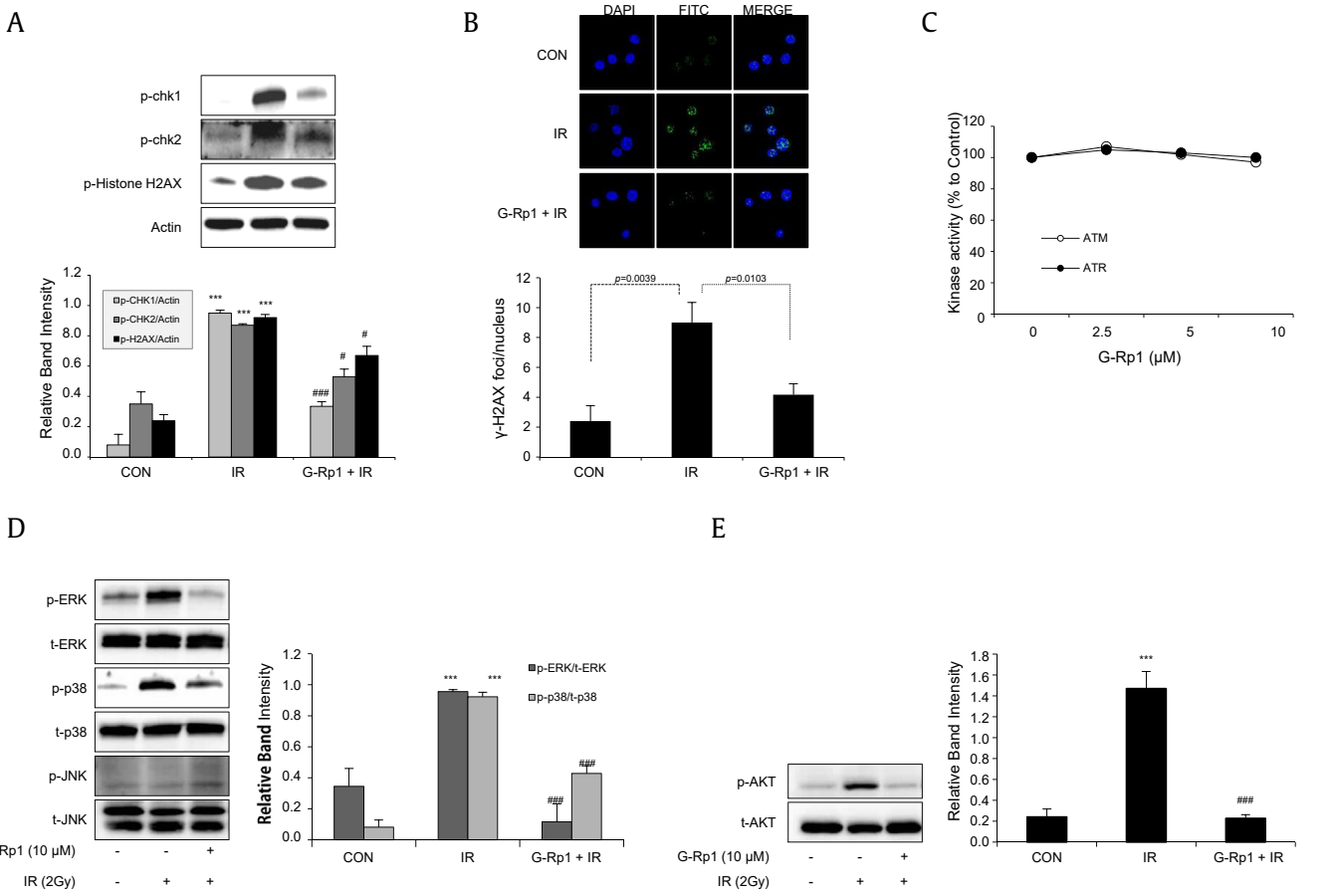

Fig. 4. Ginsenoside-Rp1 inhibits radiation-induced gene activation in J774A.1 murine macrophages. J774A.1 cells (2×10^5 cells/mL) were incubated with and without G-Rp1 (10 μM) for 30 min and irradiated (2 Gy) using a blood gamma irradiator, followed by incubation at 37°C for 10 min. (A, D, and E) The cells were lysed and Western blots were performed. (B) Ionizing radiation-induced gamma-H2AX signals were observed by confocal microscopy. (C) The effect of G-Rp1 on ATM and ATR kinases was evaluated using an *in vitro* kinase assay. The immunoblots and confocal microscopy images were analyzed using ImageJ. ***$p < 0.001$ versus control, #$p < 0.05$, ###$p < 0.001$ versus IR-treated group.

enzymatic effect on ATM and ATR activities (Fig. 4C). These results indicate that G-Rp1 may indirectly inhibit radiation-induced DNA damage-related gene activation or other molecular targets of G-Rp1.

Since MAPK activation is a key factor influencing the biological response to irradiation [6], we investigated the effect of G-Rp1 on IR-induced MAPK phosphorylation. As shown in Fig. 4D, G-Rp1 significantly inhibited ERK and p38 MAPK activation by IR, which was confirmed by the suppressive effects of the respective MAP kinase inhibitors PD98059 (ERK-inhibitor) and SB203580 (p38 MAPK-inhibitor) in irradiated cells treated with LPS to induce NO and IL-1β (data not shown). The PI3K-Akt pathway is another crucial mechanism underlying the response to irradiation [7]. Therefore, we analyzed the effect of G-Rp1 on IR-induced Akt phosphorylation. As shown in Fig. 4E, G-Rp1 inhibited IR-induced Akt activation. These results suggest that the radioprotective effect of G-Rp1 was associated with MAPK targeting and Akt activation.

Recent studies suggested that NO and IL-1β altered the phenotype of cancer cells [8,9]. We, therefore, tested the effect of CM obtained from IR and LPS-treated J774A.1 macrophages containing elevated NO and IL-1β concentrations on the malignancy of tumor cells using *in vitro* (wound healing assays, migration assays, and clonogenic assays) and *in vivo* assays (the survival of tumor-bearing mice, which is the gold standard). After the J774A.1 macrophage cells were exposed to IR and LPS with or without G-Rp1, CM was added to the corresponding CT26 colon cancer cells. As shown in Fig. 5, CM from IR and LPS-stimulated J774A.1 cells (IR + LPS) significantly enhanced wound healing (Fig. 5A) and cell migration (Fig. 5B) of CT26 colon cancer cells compared to cancer cells exposed to CM from control J774A.1 cells (CON). We also employed mouse-derived normal cell lines, TM3 and TM4 cells. CM from IR + LPS-treated J774A.1 cells did not produce phenotypic variations in the TM3 and TM4 cells (data not shown). These results suggest that the CM from IR + LPS treated macrophage cells increased cancer cell migration activities, an important indicator of tumor metastasis. The CT26 colon cancer cells exposed to CM from G-Rp1 (10 μM), IR, and LPS-treated J774A.1 cells (G-Rp1+IR + LPS) showed less wound healing and cell migration. We next employed a clonogenic assay for the same purpose. As shown in Fig. 5C, CT26 colon cancer cells exposed to the CM from G-Rp1 (10 μM), IR, and LPS-treated J774A.1 cells (G-Rp1+IR + LPS) showed decreased clonogenic ability compared to cancer cells exposed to CM from IR and LPS-treated macrophages not treated with G-Rp1 (IR + LPS). These *in vitro* malignancy indicators, as well as the tumor cell migration and clonogenic abilities of the CT26 colon cancer cells, were not influenced by direct treatment with G-Rp1 (up to 10 μM) (data not shown).

Finally, CT26 colon cancer cells treated with each CM were transplanted into BALB/c mice and the survival rates were observed (1×10^4 of CT26 colon cancer cells/mice, n = 5 per group). None of the mice implanted with CT26 colon cancer cells exposed to CM from control J774A.1 macrophages died until the end of the experiment (data not shown). As shown in Fig. 5D, 40%

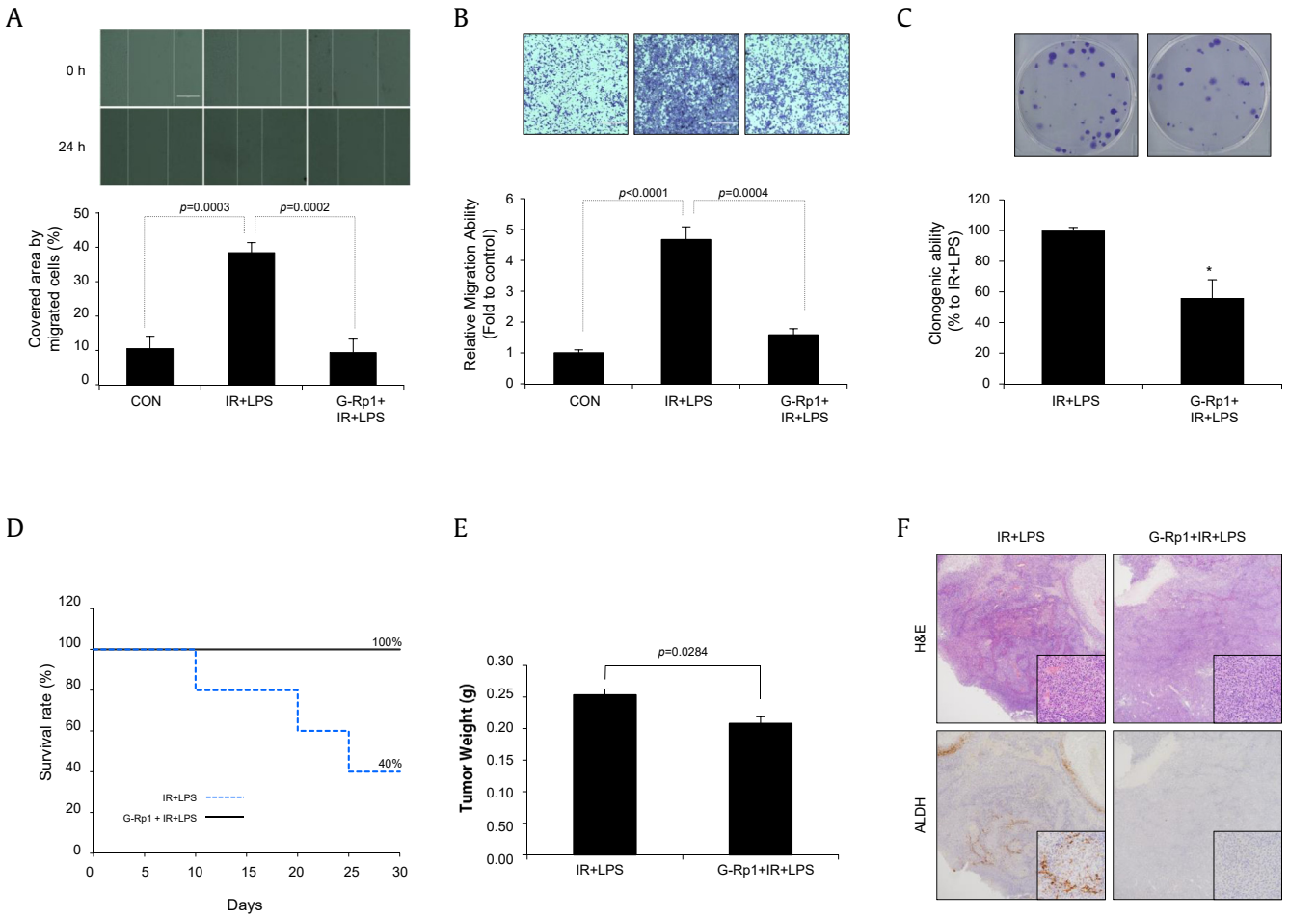

Fig. 5. Conditioned medium from G-Rp1, IR, and LPS-treated J774A.1 cells (G-Rp1 + IR + LPS) showed reduced phenotypic variation in CT26 colon cancer cells compared to conditioned medium from IR and LPS-treated J774A.1 cells (IR + LPS). After the quantification of NO and IL-1β in the conditioned medium, CT26 colon cancer cells were treated with CM from the indicated samples. To evaluate phenotypic changes in the CT26 colon cancer cells, an *in vitro* wound healing assay (A), Boyden chamber cell migration assays (B), and clonogenic assay (C) were employed. (A) Migration of the wounded CT26 cells was evaluated based on the rate of cells covering the wounded area. (Top) Representative wound healing images at 0 and 24 h. (Bottom) Quantification of the wound healing rate (200× magnification). (B) (Top) CT26 colon cancer cells that crossed the transwell membrane were stained with crystal violet (top) and quantified (bottom). Scale bar = 200 μm. (C) Representative images illustrating the clonogenic ability of the CT 26 cells treated with CM from G-Rp1+IR + LPS versus CM from IR + LPS. (Top) The colony numbers relative to the CT26 cells treated with IR + LPS CM are expressed as a percentage. (Bottom) (D) The survival rate of CT26 cell-xenografted mice treated with CM derived from G-Rp1+IR + LPS versus CM from IR + LPS. ($n = 5$ per group). (E) Comparison of tumor weights between the two groups. (F) Hematoxylin and eosin-stained images and ALDH immunohistochemical data of CT26 colon cancer cells from xenografted mice treated as indicated. All bar graphs are expressed as mean +/− SEM. *$p < 0.001$ versus IR + LPS.

of the mice transplanted with CT26 colon cancer cells treated with CM from IR and LPS-treated J774A.1 cells (IR + LPS) survived 30 days after tumor transplantation, whereas none of the mice transplanted with CT26 colon cancer cells treated with CM from G-Rp1 (10 μM), IR, and LPS-treated J774A.1 cells (G-Rp1+IR + LPS) died during this period. The tumor masses also shrank from 0.25 ± 0.01 g to 0.21 ± 0.01 g. Furthermore, hematoxylin and eosin staining and aldehyde dehydrogenases (ALDH) immunohistochemical (IHC) data revealed that the ALDH levels, which served as markers of malignancy in colon cancer cells, in the CM obtained from IR and LPS-treated J774A.1 cells with G-Rp1 (G-Rp1+IR + LPS)-treated CT26 cells were decreased compared to the CM from IR and LPS-treated J774A.1 cells (IR + LPS)-treated CT26 cells.

Despite its long history, there have been no reports on the protective effects of G-Rp1 on radiation exposure. Patient demands for overcoming the adverse pathophysiological effects of (medical) radiation exposure are increasing. The purpose of this study was to demonstrate the beneficial effects of G-Rp1 on the harmful consequences of radiation exposure through *in vitro* and *in vivo* experimental evidence. We found for the first time that G-Rp1 controlled the IR-enhanced release of NO and IL-1β in LPS-stimulated J774A.1 macrophages, thereby suppressing the phenotypic variation of CT26 colon cancer cells. Analysis of the radiation-activated intracellular signaling pathways demonstrated that G-Rp1 targeted chk1, chk2, H2AX, and their downstream activation pathways, including ERK, p38 MAPK, and AKT. Our results suggest that G-Rp1 represents a potential response to the unmet needs of radiotherapy.

Conflicts of interest

All authors have no competing interests to declare.

Acknowledgments

This work was supported by a Dongnam Institute of Radiological & Medical Sciences (DIRAMS) grant funded by the Korean government (MSIT) (Grant No. 50591-2019, and No. 50595-2019).

References

[1] Endale M, Lee WM, Kamruzzaman SM, Kim SD, Park JY, Park MH, Park TY, Park HJ, Cho JY, Rhee MH. Ginsenoside-Rp1 inhibits platelet activation and thrombus formation via impaired 210 glycoprotein VI signalling pathway, tyrosine phosphorylation and MAPK activation. Br J Pharmacol 2012;167:109–27.

[2] Cho JY, Yoo ES, Baik KU, Park MH, Han BH. In vitro inhibitory effect of protopanaxadiol ginsenosides on tumor necrosis factor (TNF)-alpha production and its modulation by known TNF-alpha antagonists. Plant a Med 2001;67:213–8.

[3] Park TY, Park MH, Shin WC, Rhee MH, Seo DW, Cho JY, Kim HM. Anti-metastatic potential of ginsenoside Rp1, a novel ginsenoside derivative. Biol Pharm Bull 2008;31:1802–5.

[4] Lee YJ, Han JY, Lee CG, Heo K, Park SI, Park YS, Kim JS, Yang KM, Lee KJ, Kim TH, et al. Korean Red Ginseng saponin fraction modulates radiation effects on lipopolysaccharide-stimulated nitric oxide production in RAW264.7 macrophage cells. J Ginseng Res 2014;38:208–14.

[5] Ibuki Y, Mizuno S, Goto R. gamma-Irradiation-induced DNA damage enhances NO production via NF-216 kappaB activation in RAW264.7 cells. Biochim Biophys Acta 2003;1593:159–67.

[6] Dent P, Yacoub A, Fisher PB, Hagan MP, Grant S. MAPK pathways in radiation responses. Oncogene 2003;22:5885–96.

[7] Zingg D, Riesterer O, Fabbro D, Glanzmann C, Bodis S, Pruschy M. Differential activation of the phosphatidylinositol 3'-kinase/Akt survival pathway by ionizing radiation in tumor and primary endothelial cells. Cancer Res 2004;64:5398–406.

[8] Kaler P, Augenlicht L, Klampfer L. 0 Macrophage-derived IL-1beta stimulates Wnt signaling and growth of colon cancer cells: a crosstalk interrupted by vitamin D3. Oncogene 2009;28:3892–902.

[9] Li Y, Wang L, Pappan L, Galliher-Beckley A, Shi J. IL-1beta promotes stemness and invasiveness of colon cancer cells through Zeb1 activation. Mol Cancer 2012;11:87.