

Effect of Korean Red Ginseng Extract on Cell Death Responses in Peroxynitrite-Treated Keratinocytes

Hyung Do Kim, Se Eun Ha, Jea Ran Kang, and Jong Kun Park*

Division of Biological Science, Wonkwang University, Iksan 570-749, Korea

Korean red ginseng (KRG) has been used worldwide as a traditional medicine for the treatment of various diseases, including cancer. In this study, we determined the effect of KRG on the responses of HaCaT cells to peroxynitrite (ONOO⁻). Cells treated with ONOO⁻ (2 mM) prior to incubation with control medium for 12 hours displayed reduced viability, as determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (viability about 48% of that of non-treated control cells). When KRG was added to the post-incubation medium, the negative effects of ONOO⁻ on cell viability were significantly reduced. Reverse transcription-polymerase chain reaction analysis indicated that KRG alone did not significantly alter p53 or “growth arrest and DNA damage” (GADD)45 mRNA levels. However, the addition of KRG to the post-incubation medium significantly and dose-dependently reduced levels of p53 and GADD45 mRNA in ONOO⁻-treated cells. Western blot analyses revealed that incubation with KRG decreased p53 and GADD45 protein levels in ONOO⁻-treated cells, relative to those in cells incubated with control medium. Collectively, these results suggest that Korean red ginseng extract protects cells against ONOO⁻-induced genotoxicity by increasing cell viability through modulating the expression of p53 signaling intermediates.

Keywords: Korea red ginseng extract, Peroxynitrous acid, Apoptosis, p53, HaCaT cell

INTRODUCTION

Peroxynitrite (ONOO⁻) is a powerful biological oxidant that is generated *in vivo* and *in vitro* by the diffusion-limited reaction of superoxide with nitric oxide [1,2]. Nitric oxide and superoxide are important mediators of tissue injury in inflammation, shock, and ischemia reperfusion injury [3,4]. Because of its oxidizing properties, ONOO⁻ can damage a wide range of cellular molecules, including DNA and proteins. Moreover, ONOO⁻ can induce the expression of keratinocyte differentiation markers [5-8].

Because damage to the DNA can result in genetic changes that may contribute to the development of cancer, cells that have sustained DNA damage constitute a

threat to multicellular organisms. Mammalian cells have evolved a range of mechanisms to minimize the risk of DNA damage. One involves growth-arrest followed by DNA repair; another is programmed cell death (apoptosis). Keratinocytes that have sustained DNA damage use the growth-arrest/DNA repair response, which is often dependent on the expression of p53 [9-14].

TP53 is an important tumor suppressor gene. While over-expression of p53 inhibits DNA synthesis and malignant transformation, its inactivation through mutation or deletion can result in malignant transformation. *TP53* is the most commonly mutated gene in human cancers. Indeed, p53 is mutated in approximately 50% of all can-

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*Corresponding author

E-mail: jkpark@wonkwang.ac.kr
Tel: +82-63-850-6200, Fax: +82-63-843-2781

cers, and more than 90% in squamous cell carcinomas of the skin. p53 target genes include the cyclin-dependent kinase inhibitor p21^{WAF1/CIP1}, MDM2, “growth arrest and DNA damage” (GADD)45, Bax, cyclin G, and proliferating cell nuclear antigen (PCNA) [3,15-21]. p53 enhances repair by transactivating its target genes. The expression of p21^{WAF1/CIP1} is induced in p53-dependent or -independent manner in response to DNA damage [16,22]. Other p53 targets that may play roles in repair include GADD45 and PCNA. GADD45 increases the accessibility of damaged chromatin through direct interaction with nucleosomes. GADD45 genes have been implicated in the signaling responses to various stressors that result in cell cycle arrest, DNA repair, cell survival or apoptosis. GADD45A is a DNA damage-inducible gene that can be regulated both p53-dependently and -independently.

Korean red ginseng (KRG) is made by steaming and drying fresh ginseng. Transformations of chemical constituents may occur during the production process. Variation among and within individual ginsenosides may be pharmacologically important, because individual ginsenosides differ in their effects on human physiology [23]. Ginseng is believed to produce various effects, including suppression of the growth and metastasis of cancer cells and activation of glutathione peroxidase and superoxide dismutase to protect against damage caused by free radicals, as well as decreasing malondialdehyde levels and increasing the activity of catalase. Also, non-saponins in red ginseng may improve learning and memory, while acidic polysaccharides activate natural killer cells and increase the production of interferons [21,24-27].

DNA in most mammalian cells is continually damaged by exogenous and endogenous agents, including reactive oxygen species and other chemicals. The DNA damage sustained can trigger the development of tumors. In the present study, we investigated the effects of KRG on cell death responses, including p53-dependent signaling, in ONOO⁻-treated cells. We discuss the possible mechanism(s) involved.

MATERIALS AND METHODS

Samples

KRG were generously provided by the Korea Ginseng Corporation.

Cell culture and DNA damage

HaCaT human keratinocytes were grown in culture

dishes in a humidified 5% CO₂ atmosphere at 37°C. The culture medium used was RPMI-1640 (Gibco Laboratories, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µM). The pH of the medium was adjusted to 7.2 to 7.4 with 10 mM Hepes buffer and 0.37% sodium bicarbonate. Cells were treated with various concentrations of ONOO⁻ (Calbiochem), diluted in PBS (pH 7.4), for 30 minutes, washed twice with PBS, and then post-incubated in control growth medium or medium containing KRG.

Cell viability assay

Cell viability was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were cultured in 96-well plates, treated with ONOO⁻, washed twice with PBS, and post-incubated in medium containing various concentrations of KRG for 12 hours. Then, the cells in each well were mixed with 10 µL of MTT solution (5 mg/mL in PBS) and the culture plates incubated for 4 hours at 37°C. Finally, DMSO was added to each well and absorbances at 570 nm were measured using a microplate reader.

Semi-quantitative reverse transcription PCR

Total RNA was extracted from cells treated with ONOO⁻ and post-incubated with KRG using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and was reverse transcribed using a First-Strand cDNA Synthesis Kit (Invitrogen). Reverse transcription-polymerase chain reactions (RT-PCR) (25 µL) containing the following were prepared: cDNA template (1 µL), a specific oligonucleotide primer pair (1 µM), and 23 µL of Taq mixture containing Taq DNA polymerase (0.5 U). The thermal conditions used were: 10 minutes at 95°C and 35 cycles of 30 seconds at 94°C, 30 seconds at 50 to 60°C, depending on the primers, and 60 seconds at 72°C. The following primers were used: p53 forward, 5'-GGG ACA GCC AAG TCT GTG-3'; p53 reverse, 5'-GGA GTC TTC CAG TGT GAT-3'; GADD45 forward, 5'-GCT CTC CTG GAC CTG GTG-3'; GADD45 reverse, 5'-CCA TGT AGC GAC TTT CCC GGC-3'; β-actin forward, 5'-ATC TGG CAC CAC ACC TTC TA-3'; β-actin reverse, 5'-CGT CAT ACT CCT GCT TGC TG-3'.

Western blotting

Lysates were prepared from cells treated with ONOO⁻ and post-incubated with KRG. Hot Laemmli lysis buffer containing PMSF (1 mM; Sigma, St. Louis, MO, USA)

was directly applied to treated cells, which were then centrifuged. Supernatants were boiled for 10 minutes, and the resulting protein samples (50 μg) were then subjected to electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gels. Proteins were electroblotted to PVDF membranes (Micron Separations, Westborough, MA, USA). Following blocking, blots were incubated with mouse anti-p53, anti-GADD45 and anti-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in TBS-T (TBS-0.05% Tween 20) for 90 minutes and then washed three times (15 minutes each) in PBS-T. They were then incubated with horseradish peroxidase-conjugated anti-mouse IgG for 1 hour. After washing, blots were incubated for 3 minutes with ECL Plus reagent (Amersham Biosciences, Piscataway, NJ, USA). Chemiluminescence was finally detected by exposure of the blots to ECL-Western film for between 10 seconds and 10 minutes.

Statistical analysis

Student's *t*-test was used to identify statistically significant differences. In the RT-PCR and Western blotting experiments, the intensity of each band was measured densitometrically. Values for each target band were normalized to that of the housekeeping gene of β -actin (Western blotting), and then compared with those of non-treated controls. Data represent the mean of at least three independent experiments.

RESULTS AND DISCUSSION

In the present study, we probed the effects of KRG on the damage responses of ONOO⁻-treated HaCaT cells. The results of the MTT assay showed that treatment for 12 hours with KRG (100 $\mu\text{g}/\text{mL}$) alone did not result in a significant reduction in cell viability (Fig. 1). Cells treated with various doses of ONOO⁻ and subsequently incubated for 12 hours with KRG showed increased cell survival compared with cells post-incubated in control medium. When cells were treated with ONOO⁻ and then incubated with control growth medium for 12 hours, cell viability decreased, to about 43% (Fig. 2). Post-incubation of ONOO⁻-treated cells in medium containing KRG, however, increased cell viability, in a KRG concentration-dependent manner.

We next measured levels of p53 and GADD45 mRNA by RT-PCR. We found that KRG (25-100 $\mu\text{g}/\text{mL}$) alone did not significantly alter p53 or GADD45 mRNA levels (data not shown). In contrast, levels of p53 and GADD45 mRNA in cells treated with

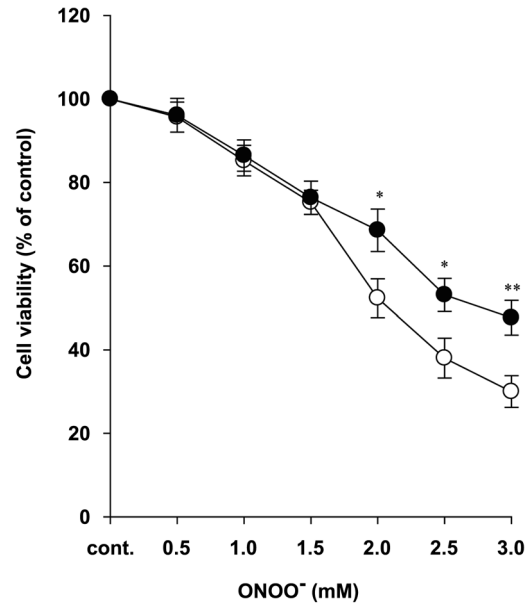


Fig. 1. Viability of HaCaT cells treated with peroxynitrite (ONOO⁻), determined by the MTT assay. Cells treated with various concentrations of ONOO⁻ for 30 minutes were post-incubated with growth medium (open circles) or Korean red ginseng extract (closed circles) for 12 hours. Data represent the mean \pm SD of triplicate experiments. **p* < 0.05 and ***p* < 0.01, vs. non-treated control.

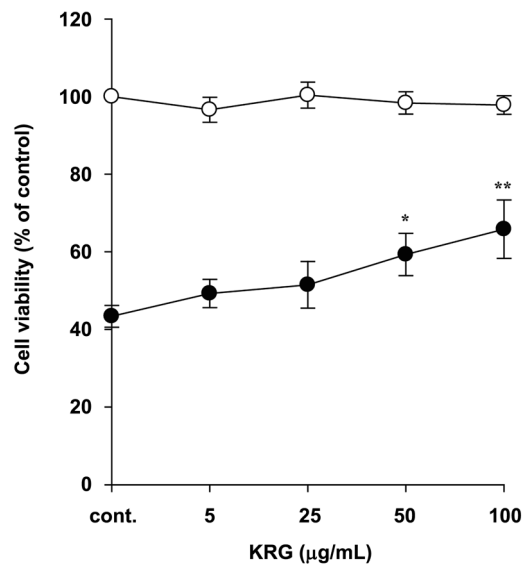


Fig. 2. Effects of KRG on the viability of HaCaT cells treated with peroxynitrite (ONOO⁻), determined by the MTT assay. Cells treated with 2 mM ONOO⁻ (closed circles) for 30 minutes or control medium (open circles) were post-incubated for 12 hours with medium containing various concentrations of KRG. Data represent the mean \pm SD of triplicate experiments. **p* < 0.05, vs. cells treated with ONOO⁻ and post-incubated with control medium for 12 hours.

ONOO⁻ and then post-incubated in growth medium for 12 hours increased, approximately 3.5-fold over those in non-treated controls (Fig. 3). Interestingly, p53 and

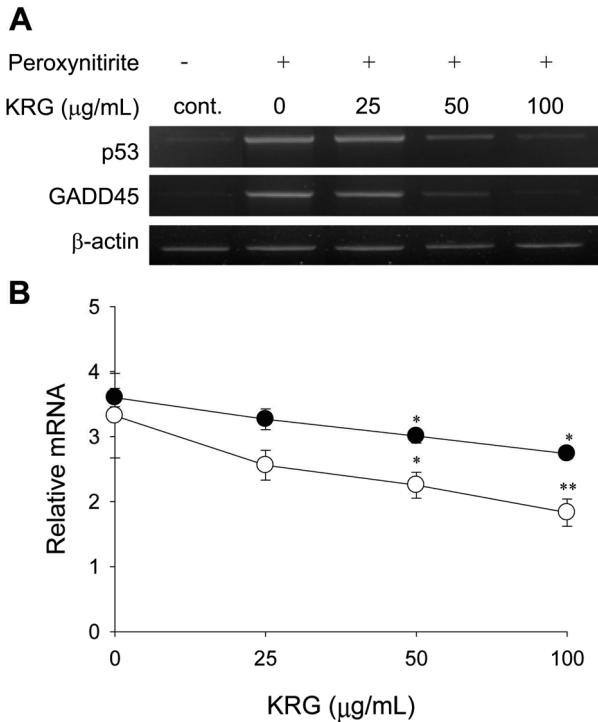


Fig. 3. Relative levels of p53 (open circles) and “growth arrest and DNA damage” (GADD)45 (closed circles) mRNA in cells treated with peroxynitrite (ONOO⁻) and post-incubated with various concentrations of KRG, as determined by reverse transcription-polymerase chain reaction. Data represent the mean \pm SD of triplicate experiments. * $p < 0.05$, vs. cells treated with ONOO⁻ and post-incubated with control medium.

GADD45 mRNA levels were down-regulated by the post-incubation of ONOO⁻-treated cells with KRG in a KRG concentration-dependent manner.

We then measured levels of p53 and GADD45 protein in cells treated with ONOO⁻ and then post-incubated for 12 hours in medium containing various concentrations of KRG (Fig. 4A). When ONOO⁻-treated cells were post-incubated with growth medium for 12 hours, levels of p53 and GADD45 increased, approximately 1.8- and 2.3-fold, respectively, over those in non-treated controls (Fig. 4B). Moreover, when ONOO⁻-treated cells were post-incubated with medium containing various concentration of KRG, p53 and GADD45 levels gradually decreased. These findings suggest that post-incubation of ONOO⁻-treated cells with KRG reduced p53 and GADD45 protein expression.

Time-course analysis showed that p53 and GADD45 protein levels in ONOO⁻-treated cells post-incubated with control medium peaked at 3 and 6 hours, respectively (Fig. 5B and 5D). Interestingly, post-incubation of ONOO⁻-treated cells with KRG caused the levels of these proteins to peak earlier, at 1 hour. Rapid up-regulation of GADD45 following ONOO⁻ treatment might play an

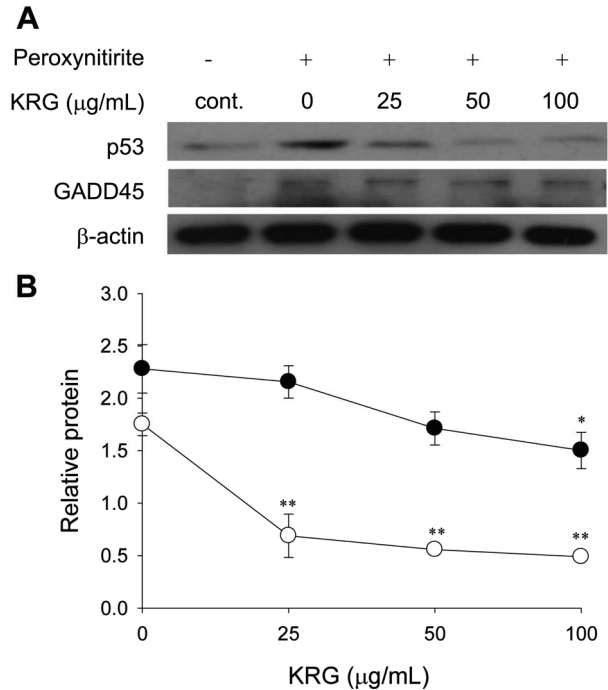


Fig. 4. Effects of post-incubation with KRG on the levels of p53 and “growth arrest and DNA damage” (GADD)45 protein in HaCaT cells treated with 2 mM peroxynitrite (ONOO⁻). (A) Cells were treated with ONOO⁻ and post-incubated for 12 hours with various concentrations of KRG (0-100 $\mu\text{g/mL}$). (B) Relative p53 (open circles) and GADD45 (closed circles) protein levels, determined by densitometric measurement of the bands produced by Western blotting, and normalized to those in non-treated controls. Data represent the mean \pm SD of triplicate experiments. * $p < 0.05$ and ** $p < 0.01$, vs. cells treated with ONOO⁻ and post-incubated with control medium.

active role in the repair of ONOO⁻-induced DNA damage. The acceleration of DNA repair by GADD45 may create a less genotoxic state, which, in turn, would not necessitate such high GADD45 expression and activity at later time points (9 to 24 hours).

We previously reported that a mixture of ginsenosides, including Rg2, enhanced DNA repair in UVB-exposed NIH3T3 cells, perhaps through the up-regulation of PCNA [28]. Unpublished observations in UVB-exposed HaCaT cells also demonstrate that the Rg2 assists the repair of UVB damage by increasing the expression of p53 and GADD45 at earlier time points. Another study showed that ginsenosides bind to DNA polymerase delta and increase its polymerase and proof-reading exonuclease activities, approximately 2-fold [15]. It remains to be determined whether KRG influences PCNA levels and/or increases DNA polymerase activity. It has been established that ginsenosides bind to the glucocorticoid and/or androgen receptors and up-regulate the expression of steroid-responsive genes [29]. Other molecules, including green tea polyphenols, retinyl esters, and the

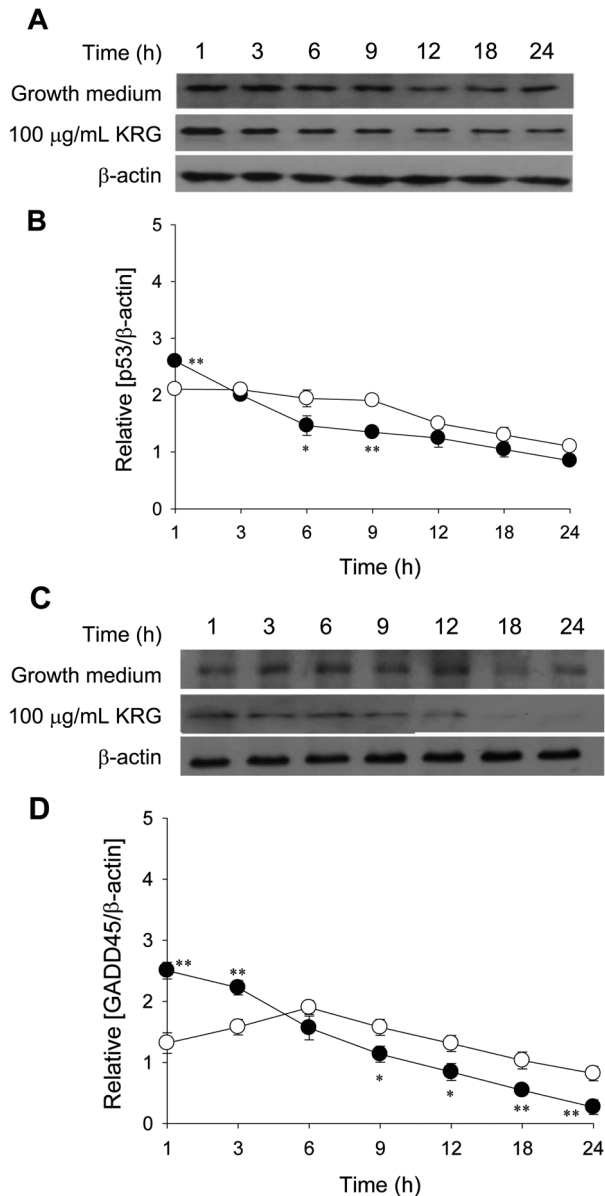


Fig. 5. Time-course analysis of the effects of post-incubation with growth medium or KRG on p53 (A,B) and “growth arrest and DNA damage” (GADD)45 (C,D) protein levels in peroxynitrite (ONOO⁻)-treated HaCaT cells. Representative Western blots (A,C) and relative p53 (B), and GADD45 (D) protein levels in cells treated with ONOO⁻ and post-incubated with control medium (open circles) or KRG (closed circles). Data represent the mean±SD of triplicate experiments. **p*<0.05 and ***p*<0.01, vs. cells post-incubated with control medium (open circles).

flavonoids genistein and silymarin, as well as synthetic UV filters, have been shown to protect the skin from UVB- or reactive oxygen species-induced DNA damage [13,30,31]. Recently, a mixture of peptides and sugars derived from plant cell walls was shown to limit age-associated molecular changes by up-regulating the expression of specific anti-aging genes in cultured skin

cells [32]. Of the protective agents described above, some have been shown to absorb UVB radiation, and to thus prevent DNA from being exposed to it. We found little evidence to suggest that Rg2 performs this kind of filtering function (unpublished results).

The up-regulation of p53 signalling intermediates, including GADD45, has been reported in human promyelocytic leukemia cells exposed to volatile organic compounds, such as benzene and toluene, suggesting that the responses of p53 pathways to genotoxic and non-genotoxic agents are disparate and complex [33]. A number of small molecules that act upstream of p53 or target the p53 protein itself to rescue p53 function have been identified recently [34]. Further studies are necessary to determine the mechanism(s) underlying the protective effects of KRG on DNA-damaged cells.

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