

Korean Red Ginseng Extract inhibits Tumor Necrosis Factor-alpha-induced Monocyte Adhesion in the Human Endothelial Cells

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Abstracts : Vascular inflammation is an important step in the development of cardiovascular disorder. Since it has not been known whether Korean red ginseng has a role to play on the vascular inflammation, we investigated the effects of Korean red ginseng extract (KRGE) on monocyte adhesion and its underlying signaling mechanism. Monocyte adhesion assay and Western blot were conducted on the human umbilical vein endothelial cells to study monocyte adhesion and the expression of adhesion molecules. Intracellular calcium was measured with Fura-2 fluorescent staining, and superoxide production was measured with lucigenin chemiluminescence in the endothelial cells. KRGE inhibits tumor necrosis factor (TNF)-alpha-induced monocyte adhesion on the endothelial cells at the range of 0.03~1 mg/ml. TNF-alpha-induced vascular cell adhesion molecule-1 and intercellular cell adhesion molecule-1 expression were inhibited by the pretreatment of KRGE in the endothelial cells. KRGE also inhibits TNF-alpha-induced intracellular calcium and the superoxide production in the endothelial cells. This study first demonstrated that KRGE inhibits TNF-alpha-induced monocyte adhesion by inhibiting the adhesion molecule expression, intracellular calcium and superoxide production in the endothelial cells. Therefore, the anti-inflammatory function of KRGE may be contributed to protecting the endothelial dysfunction in the vascular inflammatory disorders.

Key word : Korean red ginseng extract, monocyte adhesion, adhesion molecule, superoxide production.

INTRODUCTION

Atherosclerosis is an inflammatory disease¹. Expression of adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1, in the vascular endothelial cells is not only the first step but also a crucial event in the pathological process of endothelial activation². An increased expression of adhesion molecules by endothelial cells in human atherosclerotic lesions may lead to further recruitment of leukocytes to atherosclerotic sites. Vascular endothelial cells are sensitive to oxidative damage mediated by superoxide anion released from the endothelium itself and from inflammatory cells, and reactive oxygen

species (ROS) such as superoxide are implicated in the pathogenesis of cardiovascular disorders^{3, 4}.

Panax ginseng C. A. MAYER is the traditional herbal medicine that has been most popular in eastern Asia for more than 2000 years. Among the several kinds of *Panax ginseng* products, the Korean red ginseng (KRG) has the most potent multiple pharmacological actions for hypertension, diabetes, and cancer, and many Asians believe that KRG improves the conditions of weakness⁵. Possible beneficial effect of KRG on the pathogenesis of cardiovascular diseases has been suggested as the vasorelaxing effect⁶, anti-thrombotic activities⁷ and anti-hypertensive effect^{8, 9} of KRG were uncovered.

However, it has not been clear whether Korean red ginseng extract (KRGE) plays a role in the monocyte adhesion of the endothelial cells. Therefore, we investigated the effect of KRGE on the monocyte adhesion and adhe-

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sion molecule expression as well as its underlying mechanism in the cultured endothelial cells.

MATERIAL AND METHODS

Preparation of KRGE

Korean Red Ginseng extracts (KRGE, *Cheong-Kwan-Jang*) manufactured from the roots of a 6-year-old fresh Panax red ginseng (*Ginseng Radix Rubra*) were provided by the Korea Ginseng Corporation (Seoul, Korea). To use KRGE aseptically, it was first dissolved in the phosphate buffered solution at a concentration of 50 mg/ml, and then filtered with 0.2 μm microfiltration apparatus (Millipore, USA).

Cell Culture and Reagent

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics and were grown and maintained in endothelial growth medium. Cells between passages 3 and 6 were used. U937 cell lines were obtained from American Type Culture Collection (Manassa, VA, USA). Anti-VCAM-1 and anti-ICAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-labeled anti-rabbit and anti-mouse antibodies were purchased from Amersham (Buckinghamshire, UK). Human TNF-alpha and lucigenin were purchased from Sigma (St. Louis, MO, USA).

Western blot analysis

For Western blot analysis, HUVEC cells harvested in 100 μl of lysis buffer (in mmol/L: containing Tris-Cl 20, NaCl 100, EDTA 2, EGTA 2, Na_3VO_3 1, β -glycerophosphate 1, Na pyrophosphate 4, NaF 5, 1% Triton X-100, pH 7.5, and protease inhibitor cocktail). The lysate was centrifuged at 12,000 rpm for 20 min, and the supernatant was collected. Protein (40 μg) was separated by 10% SDS-PAGE and was electrotransferred onto nitrocellulose membranes. After blocking with 5% skim milk for 1 hour at room temperature, blots were incubated for overnight at 4°C with specific primary antibody (1:1000 dilution), and subsequently incubated with horseradish peroxidase-conjugated secondary antibody. Blots were developed for visualization using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL).

Monocyte adhesion assay

For quantitative adhesion assay, U937 cells were fluorescently labeled with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM)

by incubating the cells (1×10^7 cells/ml) with 1 $\mu\text{mol/L}$ BCECF-AM in RPMI-1640 medium for 30 min at 37°C and 5% CO_2 as previously described¹⁰⁻¹². HUVECs were seeded in 24-well plates to reach confluent monolayer and pretreated with KRGE for 24 hours in EGM-2 medium. Human recombinant TNF-alpha was added to appropriate wells (15 ng/ml) at 18 hour before adding labeled monocytes. Monocyte adhesion was quantified by measuring the fluorescence with excitation (485 nm) and emission (535 nm).

Measurement of superoxide production

To study the effect of KRGE on superoxide production induced by TNF-alpha, HUVEC cells were incubated with TNF-alpha for 18 hours in the presence of KRGE and in its absence. Lucigenin-enhanced chemiluminescence assay was performed to analyze the level of superoxide production as previously reported¹³⁻¹⁵. Lucigenin (*bis-N*-methylacridinium nitrate) luminesces specifically in the presence of superoxide. In brief, HUVEC (1×10^5 cells) was transferred into the scintillation vials containing Krebs-HEPES buffer (in mmol/L: NaCl 100, KCl 4.7, CaCl_2 1.9, MgSO_4 1.2, K_2HPO_4 1.03, NaHCO_3 25, Na-HEPES 20, and pH 7.4) with 5 $\mu\text{mol/L}$ lucigenin. The chemiluminescence, which occurred over the ensuing 2 min in response to the addition of 100 $\mu\text{mol/L}$ NADPH, was recorded. After subtracting a blank, the emitted light units were used as a measure of superoxide production. The values are expressed as relative light units per 1×10^5 cells (RLU/ 1×10^5 cells).

Measurement of intracellular Ca^{2+}

Fura-2 fluorescence was used as an index of $[\text{Ca}^{2+}]_i$. Fura-2 was excited by alternating 340 and 380 nm wavelength light, and Fura-2 emission was measured at 510 nm. Cells were plated onto glass coverslips in 24-well culture plates and grown to ~80% confluence. HUVEC cells were incubated in 1 $\mu\text{mol/L}$ Fura 2-AM (Molecular Probes, Cambridge, UK) for 40 min at 37°C in the dark and then left to de-esterify for 15 min in control solution. During experiments, the cells were superfused with Hank's buffered salt solution (in mmol/L: NaCl 137, KCl 5.6, MgCl_2 1, CaCl_2 2, HEPES 10, glucose 10 and pH 7.4).

Statistical Analysis

The results were expressed as mean \pm SE. Student *t* test was used to test the statistical significances between control values and drug-treated ones. For all statistical tests, a *P* value of < 0.05 was regarded as significant.

RESULTS

KRGE inhibited monocyte adhesion in the endothelial cells.

To explore whether KRGE inhibits monocyte adhesion as a marker of vascular inflammation, we examined the effect of KRGE on the monocyte adhesion to TNF-alpha-stimulated HUVECs. As shown in Fig. 1, the un-stimulated HUVECs displayed minimal monocyte adhesion. However, the monocyte adhesion was increased when HUVECs were treated with TNF-alpha. In contrast, HUVECs treated with KRGE for 18 h displayed a significant attenuation of monocyte adhesion induced by TNF-alpha (Fig. 1A). In contrast, KRGE (0.1 mg/ml) did not affect the monocyte adhesion to HUVECs in the absence of TNF-alpha, sug-

gesting that KRGE selectively prevents the endothelium-monocyte adhesion stimulated by TNF-alpha. The KRGE inhibited the monocyte adhesion in a dose dependent manner at the range of 0.03~1 mg/ml as shown in Fig. 1B.

KRGE inhibited adhesion molecule expressions

Next, we investigated whether KRGE affects the expression of adhesion molecules in endothelial cells. After the pretreatment with KRGE at 0.03~1 mg/ml, cells were exposed to 15 ng/ml of TNF-alpha for 18 hours. The levels of VCAM-1 and ICAM-1 expression were measured by western blot. As shown in Fig. 2, VCAM-1 and ICAM-1 were not detected in the un-stimulated cells, and TNF-alpha markedly induced the VCAM-1 and ICAM-1 expression. The pre-treatment with KRGE suppressed the

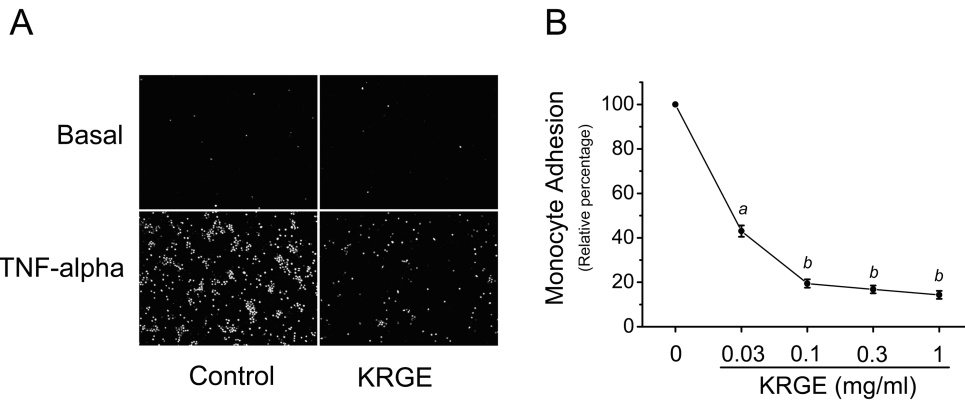


Fig. 1. Effect of Korean Red Ginseng Extract (KRGE) on the TNF-alpha-induced monocyte adhesion in the human umbilical vein endothelial cells. A. Fluorescent microscopy of monocyte adhesions. B. Summarized data of effect of KRGE (0.1 mg/ml) on the monocyte adhesion. A quantitative analysis of monocyte adhesion assay was plotted as a percentage of relative fluorescent intensity of only TNF-alpha. ^ap < 0.05 vs TNF-alpha, ^bp < 0.01 vs TNF-alpha (n=4).

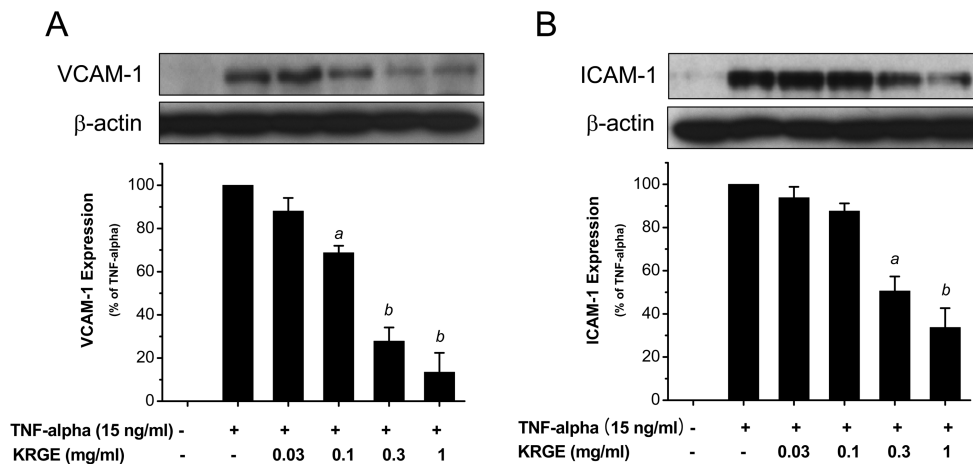


Fig. 2. Effect of Korean Red Ginseng Extract (KRGE) on the TNF-alpha-induced vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) in human umbilical vein endothelial cells. Upper panel showed Western blot data for VCAM-1(left) and ICAM-1(right). β -actin was used loading control. A quantitative analysis of adhesion molecules were plotted as a percentage expression to only TNF-alpha. ^ap < 0.05 vs TNF-alpha, ^bp < 0.01 vs TNF-alpha (n=3).

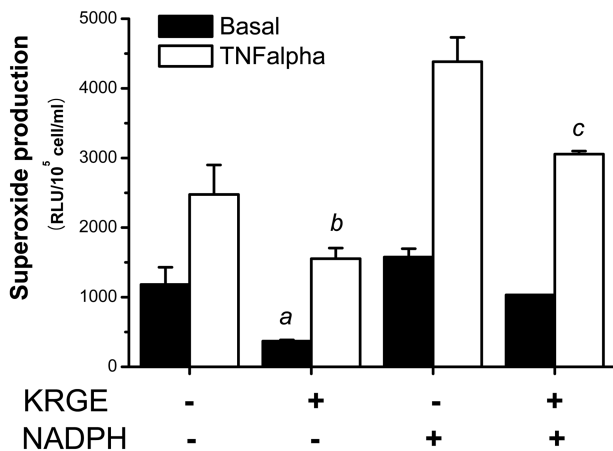


Fig. 3. Effect of Korean Red Ginseng Extract (KRGE) on TNF-alpha-induced superoxide production in the human umbilical vein endothelial cells. KRGE (1 mg/ml) inhibits both basal and TNF-alpha induced superoxide production in basal levels, and inhibits superoxide production by NADPH oxidase treatment in HUVEC. TNF-alpha was treated with 15 ng/mL for 24 h (n=3). ^ap < 0.05 vs Basal, ^bp < 0.05 vs TNF-alpha, ^cp < 0.05 vs NADPH+TNF-alpha (n=5).

TNF-alpha-induced VCAM-1 and ICAM-1 expression in a dose dependent manner (0.03~1 mg/ml).

KRGE suppressed superoxide production

Increased oxidative stress including superoxide has been shown to upregulate adhesion molecule expression¹⁶). To study whether KRGE inhibits superoxide production, we assessed NADPH-driven superoxide production with lucigenin chemiluminescence. The pretreatment of KRGE (1 mg/ml) inhibited the basal and NADPH-driven superoxide production in the endothelial cells. KRGE also inhibited NADPH-driven superoxide production in the TNF-alpha-treated endothelial cells (Fig. 3).

TNF-alpha-induced [Ca²⁺]_i was suppressed by KRGE

Intracellular calcium elevation is known as a secondary messenger to induce the vascular cell adhesion molecule-1 at the response to the TNF-alpha in the endothelial cells^{17, 18}). The TNF-alpha (15 ng/ml) significantly increased basal [Ca²⁺]_i compared to the basal state in the endothelial cells. However, the pretreatment with KRGE (1 mg/ml) inhibited the rise of TNF-alpha-induced [Ca²⁺]_i in the endothelial cells (Fig. 4).

DISCUSSION

The present study demonstrated that KRGE inhibits the TNF-alpha-induced monocyte adhesion and adhesion

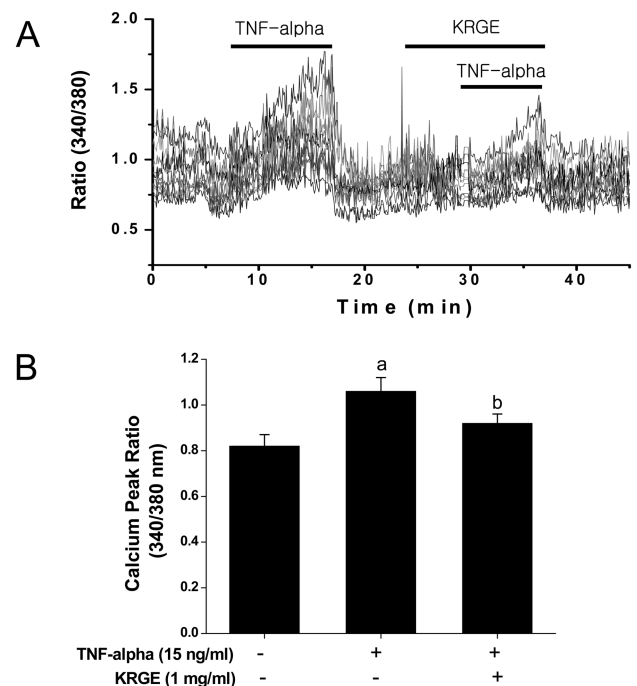


Fig. 4. Effect of Korean Red Ginseng Extract (KRGE) on the intracellular calcium concentration in the human umbilical vein endothelial cells. Change of intracellular calcium concentration was measured by fluorescent ratiometric analysis with 340/380 nm in the Fura-2 fluorescent dye loaded endothelial cells. ^ap < 0.01 vs untreated. ^bp < 0.01 vs TNF-alpha. (n=8).

molecule expressions by suppressing the superoxide production and by inhibiting the intracellular calcium influx in the cultured endothelial cells.

Atherosclerosis is the essential pathological lesion in most cardiovascular disease. The increased expression of extracellular adhesion molecules in endothelial cells is an important cause of the firm monocyte adhesion and sub-endothelial accumulation of lipids associated with the development of atherosclerosis^{1, 2}). It has been previously reported that saponin fraction of KRG decreases the superoxide production and blood pressure^{8, 19}). Therefore, we hypothesized that KRGE would inhibit the monocyte adhesion in the endothelial cells. In the present study, we showed that the KRGE produces significant inhibitory effects on the TNF-alpha-induced endothelial cell expression of adhesion molecules (ICAM-1, VCAM-1), resulting in the prevention of the monocyte adhesion to the endothelial cells. Particularly, cellular adhesion molecules are an important component in atherosclerosis and in response to vascular injury²⁰). Histological studies have demonstrated an increased endothelial expression of VCAM-1 and ICAM-1 in atherosclerotic lesions either

developing or already established^{21, 22}). This likely reflects an important function for adhesion molecules in the recruitment of monocytes to the arterial intima in the vascular injury or atherosclerosis.

Inflammatory cytokines such as TNF-alpha can activate the redox-sensitive transcription factors and induce the endothelial expression of adhesion molecules, which can be inhibited to various degrees by different antioxidants, suggesting a potential role of endogenous reactive oxygen species in atherogenesis²³). Since part of the signal transduction pathway that regulates the activation of VCAM-1 expression is redox-sensitive, the compounds with antioxidant properties may have inhibitory effects on VCAM-1 expression^{10, 24}). The evidences from experimental studies have suggested that increased vascular production of superoxide is a feature of systemic vascular disease such as vascular inflammatory disease. Among various sources of vascular superoxide such as NAD(P)H oxidases, xanthine oxidase, lipoxygenase, mitochondrial oxidases, and nitric oxide synthase, NAD(P)H oxidase appears to be the principal source of superoxide in several animal models of vascular diseases. In the present study, KRGE inhibits the basal superoxide production and the activity of NAD(P)H oxidase in the endothelial cells. The properties of KRG that reduce superoxide production may have contributed to the inhibitory action of monocyte adhesion in the endothelial cells. Our result is supported by the previous reports about the free radical scavenging activities of red ginseng^{14, 25}) and anti-NADPH-driven superoxide generation in endothelial cells¹⁹).

Calcium channels antagonists have an inhibitory action of ROS production in the endothelial cells¹⁷). The inhibition of intracellular calcium levels inhibited the TNF-alpha-induced activation of NADPH oxidase, the major source of ROS in the endothelial cells²⁶). The present study demonstrated that KRGE inhibits the TNF-alpha-induced intracellular calcium concentration in the endothelial cells. These data suggest that KRGE has a strong ability to reduce intracellular calcium by TNF-alpha in the endothelial cells. KRGE contains several kinds of ginsenosides such as Rg3, Rh2 and compound K, showing the major inhibitory action of calcium channels²⁷). Therefore, we suggest that KRGE may inhibit the upstream signaling pathways to NADPH oxidase activity via the inhibition of intracellular calcium concentration in the endothelial cells.

Taken together, it shows the KRGE plays an important role in suppressing monocyte adhesion and adhesion molecules by reducing the intracellular oxidative stress and

intracellular calcium in endothelial cells, implicating an anti-inflammatory function in the endothelial cells. Considering the biological functions of KRGE presented in this study, it is quite possible that KRGE might contribute to the preventive effects of cell damage caused by vascular inflammation and atherosclerosis. In addition, the investigation of possible effects of herbal drugs such as KRG on endothelial cells has proved an effective strategy for the development of novel pharmaceutical products for prevention and control of atherosclerotic cardiovascular diseases.

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