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Research article

Korean Red Ginseng extract induces angiogenesis through activation of glucocorticoid receptor

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ABSTRACT

Background: Our previous studies have demonstrated that ginsenoside-Rg1 can promote angiogenesis *in vitro* and *in vivo* through activation of the glucocorticoid receptor (GR). Furthermore, microRNA (miRNA) expression profiling has shown that Rg1 can modulate the expression of a subset of miRNAs to induce angiogenesis. Moreover, Rb1 was shown to be antiangiogenic through activation of a different pathway. These studies highlight the important functions of miRNAs on ginseng-regulated physiological processes. The aim of this study was to determine the angiogenic properties of Korean Red Ginseng extract (KGE).

Methods and Results: Combining *in vitro* and *in vivo* data, KGE at 500 μg/mL was found to induce angiogenesis. According to the miRNA sequencing, 484 differentially expressed miRNAs were found to be affected by KGE. Among them, angiogenic-related miRNAs; miR-15b, -23a, -214, and -377 were suppressed by KGE. Meanwhile, their corresponding angiogenic proteins were stimulated, including vascular endothelial growth factor, vascular endothelial growth factor receptor-2, endothelial nitric oxide synthase, and MET transmembrane tyrosine kinase. The miRNAs-regulated signaling pathways of KGE were then found by Cignal 45-Pathway Reporter Array, proving that KGE could activate GR.

Conclusion: KGE was found capable of inducing angiogenesis both *in vivo* and *in vitro* models through activating GR. This study provides a valuable insight into the angiogenic mechanisms depicted by KGE in relation to specific miRNAs.

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1. Introduction

Angiogenesis is the formation of new blood vessels from pre-existing blood vessels. It is involved in both physiological and pathological conditions such as embryo development [1], wound healing [2], atherosclerosis [3], and tumor growth [4]. During angiogenesis, complex cell–cell interactions and various ligand–receptor activations are involved; endothelial cells play a central role in this process [5]. Once activated by angiogenic factors, endothelial cells release proteolytic enzymes, migrate, and invade surrounding extracellular matrix, where they assemble into new blood vessels. Besides, endothelial cells are also important in regulating vascular functions, including vasodilation and blood vessel integrity. Endothelial dysfunction is associated with diverse vascular diseases, such as atherosclerosis, stroke, and hypertension [6].

Significant progress has been made in elucidating the molecular basis of endothelial functions. Recent studies have highlighted the importance of microRNAs (miRNAs) [7]. miRNAs are a group of small RNAs of approximately 18–24 bp. Although miRNAs are noncoding RNAs, they are important in regulating over 30% of gene expression at the post-transcriptional level [8]. Mature miRNAs in the cytoplasm recognize the 3′-untranslated region of target mRNAs, and their partial complementary binding to the 3′-untranslated region may lead to translational repression of the mRNA.

Ginseng (*Panax ginseng* Meyer), a traditional Chinese medicine, has been used for thousands of years. It is a slow-growing perennial herb, with large fleshy roots. Among the 11 species of ginseng, the two major species are the Asian (Chinese and Korean: *P. ginseng*)

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ginseng and American ginseng, *Panax quinquefolius*. It is well-known for its diverse benefits, includes immunomodulation, anti-inflammation, -allergy, -atherosclerosis, -hypertension, -diabetes, -stress, and -carcinogenesis, as well as wound healing [9,10].

Ginsenosides, the major bioactive ingredient in ginseng extracts, are a class of steroid glycosides and triterpene saponins. More than 100 ginsenosides have been identified from the ginseng extracts, and they are classified into protopanaxadiol and protopanaxatriol types according to their structure [11]. Among the several types of protopanaxatriol (e.g., Rf, Rh1, and Rg1) and protopanaxadiol (e.g., Rb1, Rd, and Rh2), Rg1, and Rb1 were found to be in the highest content. Our previous studies have demonstrated that ginsenoside-Rg1 can promote angiogenesis *in vitro* [12] and *in vivo* [13] through activation of the glucocorticoid receptor (GR) [14]. Furthermore, miRNA expression profiling has shown that Rg1 can modulate the expression of a subset of miRNAs to induce angiogenesis [15,16]. Moreover, Rb1 was shown to induce type I collagen expression in human dermal fibroblast by reducing the miR-25 expression [17].

These studies throw light on the important functions of miRNAs on ginseng-regulated physiological processes. By contrast, the role of miRNAs in Korean Red Ginseng extract (KGE) affecting physiological responses has not been studied so far. In this project, we aim to study the functional role of miRNAs and the underlying mechanism in KGE-regulated angiogenesis. Our results show that KGE stimulates angiogenesis *in vitro* and *in vivo* through activation of the GR.

2. Materials and methods

2.1. Reagents and chemicals

KGE was provided by Korea Ginseng Corporation (Seoul, Korea). Stock solution of KGE (50mM) was prepared in sterile water. Chemicals not specified were obtained from USB Chemicals (Cleveland, OH, USA). KGE was prepared from the roots of a 6-yr-old fresh *P. ginseng* Meyer. KGE was yielded from red ginseng water extract and the water content of the pooled extract was 36% of total weight, contained major ginsenoside-Rb1: 33.05%, Rg1: 7.95%, Re: 8.26%, Rc: 13.51%, Rb2: 11.51%, Rd: 4.04%, Rf: 5.51%, Rh1: 4.49%, Rg2S: 5.51%, and Rg3S: 6.18%.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs; Lonza, Walkersville, MD, USA), were maintained in medium M199 supplemented with heparin (90 mg/L), heat-inactivated fetal bovine serum (20%, v/v), endothelial cell growth supplement (20 μg/mL), and penicillin and streptomycin (1%, v/v). They were kept at 37°C in humidified air with 5% CO_2 and were used within passages 2–8. The cells were seeded overnight and treated with KGE in M199 containing fetal bovine serum (1%, v/v) and endothelial cell growth supplement (10 μg/mL).

2.3. Cell proliferation assay

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT) kit (USB). Equal numbers of HUVECs (1×10^4 cells/well) were seeded onto 96-well plates and incubated overnight. After the indicated time, cells were incubated with MTT solution (0.5 mg/mL) in assay medium for 4 h. Then the residual MTT was removed and the crystals were dissolved by incubation with DMSO solution for color development. The absorbance at wavelengths 450 nm and 690 nm (reference) were measured using a microplate reader (ELx800; Biotek, Winooski, VT, USA).

2.4. Cell migration assay

To evaluate the migration ability of the cells, HUVECs (3×10^4 cells/well) were seeded onto 96-well plates and incubated overnight. A denuded cell area was created by scratching the 100% confluent cell monolayer using a mechanical wounder [18]. After scratching, culture medium was replaced with fresh medium with or without KGE, and images of each well at the beginning (At_0) and after 16 h (At_{16}) were captured. The scratched area was measured using the Image J software (http://rsb.onfo.nih.gov). The migration of cells toward the denuded area was expressed as the percentage of recovery:

$$\text{Percentage of recovery} = (At_0 - At_{16} / At_0) \times 100\%. \quad (1)$$

2.5. Endothelial tube formation assay

A 96-well plate pre-coated with growth factor-reduced Matrigel (BD Bioscience, San Jose, CA, USA) was allowed to solidify at 37°C for 1 h. HUVECs (3×10^4 cells/well) were then plated on the Matrigel substratum and cultured in medium with or without KGE. Tube network in each well were captured after 8 h, the angiogenic activities were determined by counting the number of branch points of the formed tubes in each well.

2.6. Zebrafish endogenous alkaline phosphatase-based vascular staining

Zebrafish embryos (24 h postfertilization) were dechorionated by pronase (2 mg/mL) for 15 min. The embryos were then incubated with various concentrations of KGE in water containing 1-phenyl-2-thiourea at 28.5°C for another 48 h. Embryos (72 h postfertilization) were euthanized, and alkaline phosphatase activity were assayed after fixation for 30 min at 4°C in 4% paraformaldehyde. Then, fish embryos were treated with ethanol (50% and 100%) for 5 min, respectively. Dehydrated embryos were then incubated in pre-chilled acetone for 30 mins at −20°C and rinsed with phosphate-buffered saline with 0.1% Tween-20. For staining, embryos were equilibrated with alkaline phosphatase buffer at room temperature for 15 min and subsequently stained with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phospate (AMRESCO, Solon, OH, USA) at room temperature for 30 min in dark. The subintestinal vessels (SIV) basket of the stained zebrafish was examined under stereomicroscope (Olympus SZX16) with attached digital camera (Olympus DP71; Olympus America, San Jose, CA, USA). Areas of SIVs were quantified by Image J software (http://rsb.onfo.nih.gov).

2.7. Western blot analysis

After treatment, cells were washed twice with ice-cold phosphate-buffered saline and lysed in lysis buffer (Novagen, Madison, WI, USA) containing protease (0.5%, v/v) and phosphatase inhibitor cocktails (0.5%, v/v; Calbiochem, Billerica, MA, USA). The cells were harvested by scraping, and the cell lysate was collected after centrifugation. The protein concentration of the cell lysates was determined by the DC protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated by 10% SDS-PAGE

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