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Research article

Effects of gintonin on the proliferation, migration, and tube formation of human umbilical-vein endothelial cells: involvement of lysophosphatidic-acid receptors and vascular-endothelial-growth-factor signaling

Sung-Hee Hwang [1,☆], Byung-Hwan Lee [2,☆], Sun-Hye Choi [2], Hyeon-Joong Kim [2], Kyung Jong Won [3], Hwan Myung Lee [4], Hyewon Rhim [5], Hyoung-Chun Kim [6], Seung-Yeol Nah [2,*]

[1] Department of Pharmaceutical Engineering, College of Health Sciences, Sangji University, Wonju, South Korea
[2] Department of Physiology, College of Veterinary Medicine and Bio/Molecular Informatics Center, Konkuk University, Seoul, South Korea
[3] Department of Physiology, School of Medicine, Konkuk University, Chungju, South Korea
[4] Department of Cosmetic Science, College of Natural Science, Hoseo University, Asan, South Korea
[5] Center for Neuroscience, Korea Institute of Science and Technology, Seoul, South Korea
[6] Neuropsychopharmacology and Toxicology Program, College of Pharmacy, Kangwon National University, Chunchon, South Korea

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ABSTRACT

Background: Ginseng extracts are known to have angiogenic effects. However, to date, only limited information is available on the molecular mechanism underlying the angiogenic effects and the main components of ginseng that exert these effects. Human umbilical-vein endothelial cells (HUVECs) are used as an *in vitro* model for screening therapeutic agents that promote angiogenesis and wound healing. We recently isolated gintonin, a novel ginseng-derived lysophosphatidic acid (LPA) receptor ligand, from ginseng. LPA plays a key role in angiogenesis and wound healing.
Methods: In the present study, we investigated the *in vitro* effects of gintonin on proliferation, migration, and tube formation of HUVECs, which express endogenous LPA1/3 receptors.
Results: Gintonin stimulated proliferation and migration of HUVECs. The LPA1/3 receptor antagonist, Ki16425, short interfering RNA against LPA1 or LPA3 receptor, and the Rho kinase inhibitor, Y-27632, significantly decreased the gintonin-induced proliferation, migration, and tube formation of HUVECs, which indicates the involvement of LPA receptors and Rho kinase activation. Further, gintonin increased the release of vascular endothelial growth factors from HUVECs. The cyclooxygenase-2 inhibitor NS-398, nuclear factor kappa B inhibitor BAY11-7085, and c-Jun N-terminal kinase inhibitor SP600125 blocked the gintonin-induced migration, which shows the involvement of cyclooxygenase-2, nuclear factor kappa B, and c-Jun N-terminal kinase signaling.
Conclusion: The gintonin-mediated proliferation, migration, and vascular-endothelial-growth-factor release in HUVECs via LPA-receptor activation may be one of *in vitro* mechanisms underlying ginseng-induced angiogenic and wound-healing effects.

* Corresponding author. Ginsentology Research Laboratory and Department of Physiology, College of Veterinary Medicine, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, South Korea.
E-mail address: synah@konkuk.ac.kr (S.-Y. Nah).

☆ These authors contributed equally to this work.

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1. Introduction

Wound healing is a physiological process that involves cell proliferation and migration to restore normal state after injury of the skin, blood vessels, and other tissues. This process includes hemostasis, inflammation, angiogenesis, collagen deposition, epithelialization, and remodeling [1]. Angiogenesis is a critical component in wound healing and involves a series of steps, including proliferation and migration of endothelial cells [1,2]. Endothelial dysfunction causes impairment in wound healing and angiogenesis. The therapeutic effects of angiogenesis have been investigated in repairing and minimizing tissue damage due to cardiovascular diseases, such as coronary heart diseases and peripheral arterial diseases, and wound-healing disorders [3–6].

Ginseng, the root of *Panax ginseng*, is a traditional herbal medicine used as a tonic for invigorating the body or for alleviating a variety of diseases, including cardiovascular diseases, rheumatoid arthritis, diabetes mellitus, and cancer [7–10]. Ginseng contains several bioactive components, such as ginsenosides, acidic polysaccharides, and other unidentified components. Previous studies have shown that the ginseng extract and the ginseng saponin fraction, which contain a mixture of ginsenosides and other unidentified ingredients, stimulate angiogenesis [9,11,12]. Each of the ginsenosides has different effects on angiogenesis or wound healing. While ginsenoside Rg1 stimulates angiogenesis [12,13], ginsenosides Rg3 and compound K inhibit *in vitro* angiogenesis [14,15]. Although ginsenoside Rb1 and Rd seem to stimulate wound healing of the skin [16,17], the angiogenic effects of those ginsenosides are controversial depending on experimental models [16,18]. In addition, ginseng extracts and the total saponin fraction of ginseng include additional unidentified ingredients. Therefore, the active component responsible for the effects of ginseng remains to be clarified.

Recently, we isolated gintonin, a lysophosphatidic-acid (LPA) receptor ligand, from ginseng [19,20]. Gintonin consists of a complex of ginseng proteins and LPAs, and potently activates LPA receptors in animal cells. LPA-receptor activation plays a role in diverse cellular effects, including proliferation and migration of cells, vascular development, and neurite retraction [21]. LPA-receptor-mediated cellular effects are further coupled to biological activities, such as brain development, angiogenesis, embryo implantation, spermatogenesis, and wound healing [21].

Human umbilical-vein endothelial cells (HUVECs) express endogenous LPA-receptor subtypes, LPA1 and LPA3 [22]. LPA induces proliferation and migration of HUVECs, and silencing of LPA1/3 by short interfering RNA (siRNA) markedly suppresses the LPA-induced proliferation and migration of HUVECs, which are essential steps for angiogenesis [23]. In addition, LPA stimulates the release of vascular endothelial growth factor (VEGF), which in turn facilitates the angiogenic processes [24]. In addition, cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NF-κB) are involved in the increased release of VEGF by various stimulators [25,26]. On the basis of the findings reported previously, we assumed that activation of the LPA receptors by gintonin can be a molecular basis of ginseng-extract-induced angiogenesis.

In the present study, we examined the *in vitro* angiogenic effects of gintonin in HUVECs. Gintonin stimulated the proliferation, migration, and tube formation of HUVECs. In particular, we discussed the molecular mechanisms underlying gintonin-induced wound-healing effect with the evidence of LPA-receptor activation, VEGF release, and activation of COX-2 and NF-κB by gintonin in HUVECs. Our results show that gintonin can induce *in vitro* angiogenesis and wound healing through the activation of LPA receptors and VEGF signaling pathways.

2. Materials and Methods

2.1. Materials

Crude gintonin was isolated from P. ginseng as described previously [19]. Gintonin is a glycolipoprotein containing ginseng protein complexed with LPA [20]. Ginsenosides were purchased from the LKT Laboratories Inc. (St. Paul, MN, USA). VEGF, basic fibroblast growth factor, and Quantikine human VEGF immunoassay kit were purchased from R&D Systems (Minneapolis, MN, USA). M199 medium and 0.1% gelatin solution were purchased from WelGENE (Daegu-si, Korea). Matrigel (growth factor reduced) and collagen type 1 were purchased from BD Biosciences (Bedford, MA, USA). All other reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

HUVECs were isolated from human umbilical cord veins by collagenase treatment as described previously [27], and cultured in M199 medium supplemented with 20% (volume/volume) fetal bovine serum (FBS), 5 units/mL heparin, 3 ng/mL basic fibroblast growth factor, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cultures were maintained at 37°C in humidified conditions under 5% CO_2. The cells at passages 2–7 were used in all the experiments.

2.3. Cell proliferation

Proliferation of HUVECs was determined using a sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt (XTT)-based assay, which measures the cell viability based on the activity of mitochondrial enzyme [28]. Cells were seeded at 3×10^3 cells per well into 96-well plates coated with 0.1% gelatin solution. After 24 h, the cells were washed with M199 medium and incubated for 6 h with M199 containing 1% FBS. The cells were washed with fresh M199 (1% FBS) again, and incubated with gintonin, ginsenosides, or VEGF at the specific concentrations. Inhibitors were added 30 min before the incubation period. After the indicated incubation time, cell proliferation was assessed using the XTT assay as described previously [28]. The culture medium of cells in each well of the 96-well plate was replaced with 200 μL of serum-free medium without phenol red. Then, 50 μL of XTT reaction solution (containing 1 mg/mL XTT and 0.0306 mg/mL phenazine methosulfate) was added to each well. After incubation for 2 h, the absorbance was measured at 450 nm, which correlates with the cell viability.

2.4. Migration assay

The chemotactic motility of HUVECs was measured using the modified Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) as described previously [20]. Briefly, a polycarbonate membrane with an 8-μm pore size (Neuro Probe) was coated with 0.1 mg/mL of collagen type I from the rat tail (BD Biosciences, San Jose, CA, USA). Gintonin, VEGF, or ginsenosides in M199 (0.1% bovine serum albumin) were added to the lower chambers. The Boyden chamber was assembled by laying the membrane and the top chamber on the lower chambers. Cells (5×10^4 cells/well) were loaded to the top chambers and incubated for 70–80 min at 37°C. In some experiments, the cells were placed in the lower chambers with or without the inhibitors. The Boyden chamber was assembled and placed upside down, and incubated for 60 min. Then, the chamber was returned to the upright position, and gintonin or VEGF in M199 (0.1% bovine serum albumin) was added to the upper chambers, followed by incubation for an additional 120 min. The cells on the

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