Fermented Ginseng with Bifidobacterium Inhibits Angiogenesis of Human Umbilical Endothelial Cells \textit{in vitro} and \textit{in vivo}

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Abstract — Ginseng is a widely-used alternative medicine for the treatment of cancer, diabetes, and cardiovascular diseases. Active components of \textit{P. ginseng}, absorbed through gastrointestinal tract are the fermented ginsenosides by intestinal microorganisms. In the present study, we investigated the inhibitory effects of fermented ginseng with \textit{Bifidobacterium} (FGb) on the angiogenesis by analyzing \textit{in vitro} tube formation and invasion assay using human umbilical vein endothelial cells (HUVECs), and \textit{in vivo} angiogenesis using chick chorioallantoic membrane (CAM) assay. Treatment with FGb inhibited tube-like structure formation in a concentration-dependent manner. In addition, FGb significantly suppressed HUVEC invasion through Matrigel. Moreover, FGb dose-dependently inhibited VEGF-induced angiogenesis in a CAM assay. These results suggest that FGb is a valuable anti-angiogenic remedy.

Key words □ Fermenta ginseng, Angiogenesis, Tube formation, Migration, Chick chorioallantoic membrane

INTRODUCTION

Ginseng, the root of \textit{Panax ginseng} C.A. Meyer, has been used in traditional medicine in Korea and East Asian countries for more than 1000 years. The effects of ginseng include general ‘tonic’, anti-fatigue, anti-stress, immunomodulatory, and anti-cancers (Shin \textit{et al.}, 2000; Yue \textit{et al.}, 2006). Nowadays, it is one of the world-wide alternative medicines for cancer, diabetes, and cardiovascular diseases. Up to now, more than 30 different ginsenosides have been isolated, each with a different set of properties. The ginsenosides can be subdivided as protopanaxadiol-containing compounds such as Rg\textsubscript{3} and Rh\textsubscript{2}, and protopanaxatriol-containing ones such as Rg\textsubscript{1} and Rh\textsubscript{1}. Among them, Rg\textsubscript{1}, the most abundant and active component, has been reported to promote angiogenesis \textit{in vitro} and \textit{in vivo} (Sengupta \textit{et al.}, 2004; Liang \textit{et al.}, 2005). In addition, Rg\textsubscript{1} has been reported to induce vascular endothelial growth factor (VEGF) in human endothelial cells (Leung \textit{et al.}, 2006). In contrast, Rg\textsubscript{3} has been reported to exhibit \textit{in vitro} and \textit{in vivo} anti-carcinogenic, antimetastatic and anti-angiogenic effects (Mochizuki \textit{et al.}, 1995; Shinkai \textit{et al.}, 1996; Liu \textit{et al.}, 2000; Yun \textit{et al.}, 2001; Jin \textit{et al.}, 2006). In spite of various activities of ginsenosides, oral bioavailability of intact ginsenosides is extremely low since intact ginsenosides are hardly decomposed by gastric juice and poorly absorbed (Karikutta \textit{et al.}, 1991; Tanizawa \textit{et al.}, 1993; Xu \textit{et al.}, 2003). However, protopanaxadiol-type ginsenosides such as Rh\textsubscript{1}, Rh\textsubscript{2}, and Re are metabolized by colonic bacteria to 20-O-(β-D-glucopyranosyl)-20(S)-protopanaxadiol that is easily absorbed. In addition, it is known that an inhibitory effect on tumor cell invasion and angiogenesis is induced by the metabolite (Mochizuki \textit{et al.}, 1995). Furthermore, it has been demonstrated that the compound mediating \textit{in vivo} antimetastatic effects of ginsenosides is the metabolite, 20-O-(β-D-glucopyranosyl)-20(S)-protopanaxadiol (Hasegawa \textit{et al.}, 1997). It is now well recognized that the deglycosylation process of ginsenoside by colonic bacteria is crucial for its pharmacological expression.

Fermented Ginseng with \textit{Bifidobacterium} (FGb) is obtained by fermenting ginseng power (sterilized at 121°C with pressure for 15 min) with \textit{Bifidobacterium} at 37°C for 7 days. Contrast to the intestinal bacteria, \textit{Bifidobacterium} is beneficial to health and do not generate bad smell. In addition, FGb is known to contain 20-O-(β-D-glucopyranosyl)-20(S)-protopanaxadiol and 20(S)-protopanaxatriol, the biologically active compounds for
angiogenesis is the physiological process in the body that involves the growth of new blood vessels from pre-existing vessels. In normal physiology, it is essential in reproduction, embryonic development, menstrual cycle and wound repair (Mousa et al., 2005; Rosenblatt and Azar, 2006). However, inappropriate angiogenesis is involved in the pathogenesis of many chronic diseases including tumor (Lin et al., 2006). The angiogenic process is composed of complex and diverse cellular actions such as extracellular matrix degradation, and proliferation, migration, and differentiation of endothelial cells, which is tightly regulated by angiogenic stimulators and inhibitors. Among the factors, VEGF is well known to play a key role in regulating normal and abnormal angiogenesis by stimulating endothelial cell migration and proliferation (Isner and Asahara, 1999). Tumor-induced angiogenesis is also driven by VEGF released by tumor cells in high metabolic demands, and is essential for the growth of tumor (Folkman, 1971; Blouw et al., 2003).

Each of the angiogenesis elements, basement membrane disruption, cell migration, cell proliferation, and tube formation can be a target for angiogenesis intervention therapy, and thus for cancer therapy.

Although the ginseng colonic metabolite, 20-O-β-D-glucopyranosyl)-20(S)-protopanaxadiol, has been reported to inhibit tumor cell growth and anti-metastasis (Hasegawa et al., 1995; 1997a), the anti-angiogenic effect of the metabolite, 20-O-β-D-glucopyranosyl)-20(S)-protopanaxadiol, is not clearly reported yet. In the present study, we investigated the inhibitory effects of FGb on the VEGF-induced angiogenesis by analyzing in vitro tube formation and invasion assay using HUVECs, and in vivo angiogenesis using chick chorioallantoic membrane (CAM) assay.

MATERIALS AND METHODS

Materials

Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). EGM-2 bullet kit which contains an endothelial cell basal medium-2 (EBM-2 medium) and EGM-2 singlequots (supplements and growth factors) were purchased from Cambrex (San Diego, CA, U.S.A.). Glutamax and cortisone acetate were purchased from Gibco (Grand Island, NY, U.S.A.) and Sigma (St. Louis, MO, U.S.A.), respectively. Matrigel basement membrane matrix was purchased from BD Biosciences (San Diego, CA, U.S.A.). FGb was generously obtained from WonPharm Co., LTD (Iksum, Korea).

Cell culture

HUVECs were cultured in a gelatin-coated flask at 37°C, 5% CO₂ with EBM-2 medium supplemented with 2% fetal bovine serum (FBS), ascorbic acid, hydrocortisone, human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), human epidermal growth factor (hEGF), long R insulin-like growth factor-1 (RL-IGF-1), gentamicin sulfate (GA-1000) and heparin. HUVECs were used between passages 2 and 6 for the whole experiment.

Tube formation assay

The tube formation assays were performed on 24-well plates coated with 100 µl of matrigel basement membrane matrix per well and polymerized at 37°C for 30 min. HUVECs were suspended in 2% FBS and other supplement containing EBM-2 medium. Cells were plated on matrigel at a density of 5 × 10⁴ cells per well, and test compound was added to the culture medium. After 14 h, four fields were randomly selected from each culture and photographed with a CCD camera (TE2000-U, Nikon, Japan).

Cell invasion assay

The upper and lower parts of transwell (Corning Costar, Cambridge, MA, U.S.A.) inserts were coated with 40 µl of matrigel (1.0 mg/ml) and 40 µl of type I collagen (0.5 mg/ml), respectively. The transwell inserts were put in FBS-free EBM-2-filled 24-well plate. To the lower chamber, 5% FBS was added. HUVECs (5 × 10⁴ cells/chamber) were plated on the matrigel-coated transwell, and then, treated with FGb for 24 h. After 24 h, the inserts were taken out and the inner surface of the insert was carefully wiped out using a cotton swab. The cells that had invaded to the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. Cells in four random fields of the insert were counted under a light microscope (TMS, Nikon, Japan).

Chick chorioallantoic membrane (CAM) Assay

In vivo neovascularization was examined by the method previously described by Mousa et al. (2005). Briefly, 10-day-old embryos were purchased from Baeul-ja (Cheongsong, Korea), a farm, and were incubated at 37°C with 55% relative humidity. A small hole was punctured in the shell concealing the air sac using a hypodermic needle. A second hole was punctured in the shell on the broadside of the egg directly over avascular portion