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Angiogenic activity of sesamin through the activation of multiple signal pathways

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ABSTRACT

The natural product sesamin has been known to act as a potent antioxidant and prevent endothelial dysfunction. We here found that sesamin increased *in vitro* angiogenic processes, such as endothelial cell proliferation, migration, and tube formation, as well as neovascularization in an animal model. This compound elicited the activation of multiple angiogenic signal modulators, such as ERK, Akt, endothelial nitric oxide synthase (eNOS), NO production, FAK, and p38 MAPK, but not Src. The MEK inhibitor PD98059 and the PI3K inhibitor Wortmannin specifically inhibited sesamin-induced activation of the ERK and Akt/eNOS pathways. These inhibitors reduced angiogenic events, with high specificity for MEK/ERK-dependent cell proliferation and migration and PI3K/Akt-mediated tube formation. Moreover, inhibition of p38 MAPK effectively inhibited sesamin-induced cell migration. The angiogenic activity of sesamin was not associated with VEGF expression. Furthermore, this compound did not induce vascular permeability and upregulated ICAM-1 and VCAM-1 expression, which are hallmarks of vascular inflammation. These results suggest that sesamin stimulates angiogenesis *in vitro* and *in vivo* through the activation of MEK/ERK-, PI3K/Akt/eNOS-, $p125^{FAK}$-, and p38 MAPK-dependent pathways, without increasing vascular inflammation, and may be used for treating ischemic diseases and tissue regeneration.

Introduction

Angiogenesis is a process that forms new blood vessels and is tightly controlled by a balance between positive and negative factors [1]. The angiogenic process requires a number of different sequential steps including endothelial cell proliferation, migration, and tube formation. Newly formed blood vessels provide a route for supplying metabolic requirements such as nutrients, growth factors, and oxygen to the site of hypoxic or ischemic tissues and organs caused by defective blood circulation, and functionally improves ischemia-associated tissue damage and injury. Drugs and techniques that promote neovascularization serve as a useful strategy for treating ischemic diseases. The well-known angiogenic activator vascular endothelial growth factor (VEGF) has been used as a therapeutic drug for several human diseases such as myocardial infarction, cerebral ischemic injury, limb ischemia, and wound healing [2]. Thus, the research for development and identification of new angiogenic inducers has recently gained a growing interest.

Sesamin, a potent antioxidant, is the most abundant lignan in sesame seed oil [3] and is found in several medicinal herbs, including *Acanthopanax senticosus* [4]. Sesamin has been shown to elicit various pharmacological effects, such as chemoprevention, anti-inflammation, anti-hypertension, and protection against oxidative liver damage [5–7]. This compound improves endothelial dysfunction and endothelium-dependent vascular relaxation in an animal model [8]. Furthermore, sesamin has been shown to increase nitric oxide (NO) production via the elevation of the endothelial nitric oxide synthase (eNOS) protein in cultured endothelial cells and mouse aortic tissues, resulting in cGMP-dependent vasorelaxation and improvement of vascular function [9,10]. These evidences suggest that sesamin can promote endothelial cell function, which is closely associated with angiogenesis. However, the effect of sesamin on angiogenesis and its underlying signal cascade has not been studied.

We here examined the effect of sesamin on *in vitro* and *in vivo* angiogenesis and its angiogenic signal mechanism in cultured human umbilical vein endothelial cells (HUVECs). We found that

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sesamin promoted *in vitro* and *in vivo* angiogenesis by directly activating the multiple signal pathways such as MAPKs and Akt/eNOS, without altering the expression of VEGF and vascular adhesion molecules. Herein, we suggest that sesamin may possess therapeutic potential for the treatment of various ischemic diseases caused by defective blood circulation as well as delayed wound healing.

Materials and methods

Materials. Heparin, streptomycin, penicillin, and medium 199 (M199) were purchased from Invitrogen Life Technologies (Carlsbad, CA). The following reagents: VEGF was purchased from Upstate Biotechnology (Lake Placid, NY); TNF-α and neutralizing antibody for VEGF from R&D Systems (Minneapolis, MN); antibodies against phospho-ERK, phospho-Akt, phosphor-Src, and phospho-p38 from Cell Signaling Technology (Danvers, MA); antibodies against $p125^{FAK}$, ICAM-1, and VCAM-1 from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against phospho-eNOS as well as Matrigel from BD (Franklin Lakes, NJ). Sesamin was isolated from dried stems of *Acanthopnax divaricatus* var. *albeofructus* and confirmed its structure in comparison with the previous report [11]. The purity of sesamin is more than 98% in HPLC analysis. All other reagents were purchased from Sigma (St. Louis, MO) unless indicated otherwise.

In vitro angiogenesis assay. HUVECs were isolated from human umbilical veins by collagenase digestion, as described previously [12], and used in passages 3–7 for this experiment. HUVEC proliferation was determined by testing mitochondrial enzyme function according to the colorimetric method using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously [13]. HUVEC migration was assayed using Transwell plates (Corning Costar, Cambridge MA) with 6.5-mm diameter polycarbonate filters (8-μm pore size) as described previously [12]. Tube-like structure formation of HUVECs was determined in growth factor-reduced Matrigel as described previously [12].

Intravital microscopy assay. For the measurement of *in vivo* angiogenesis by intravital microscopy, abdominal wall windows were implanted into male BALB/c mice (6- to 8-week-old) as previously described [13]. Matrigel (100 μl) containing sesamin (20 nmol) or VEGF (100 ng) was applied to the window-inner space. After 4 days, animals were anesthetized and injected intravenously with 50 μl of 25 mg/ml fluorescein isothiocyanate-labeled dextran (M_W 250,000) via tail vein. Neovascularization was recorded using a 100-W mercury lamp and filter set for blue light (440–475 nm for excitation; 530–550 nm for emission) under a Zeiss Axiovert 200M microscope. The assay was scored from 0 (least positive) to 5 (most positive) in a double-blinded manner.

Western blotting. Cell lysates (40 μg protein) were separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with antibodies against target proteins for 2 h following pre-hybridization with bovine serum albumin. The signal intensities of immunoreactive bands were visualized by an enhanced chemiluminescence system, as described previously [12].

$p125^{FAK}$ phosphorylation assay. HUVECs were lysed in 1 ml of lysis buffer [20 mM Tris/HCl (pH 8.0), 2 mM EDTA, 137 mM NaCl, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 1% Triton X-100]. Lysates were clarified by centrifugation at 15,000*g* for 10 min, and the resulting supernatants were immunoprecipitated with anti-$p125^{FAK}$ antibody at 4 °C for overnight, followed by the addition of protein A-agarose beads at 4 °C for 1 h. Immunoprecipitates were washed twice with lysis buffer, solubilized in SDS–PAGE sample buffer, and further analyzed by Western blotting using anti-PY20.

Reverse transcriptase-polymerase chain reaction (RT-PCR). The level of VEGF mRNA was determined by RT-PCR [12]. In brief, total RNAs were obtained from HUVECs with a Trizol Reagent kit (Invitrogen, Carlsbad, CA). RNA (5 μg) was reversely transcribed using reverse transcriptase and the resultant cDNAs were amplified by PCR with primers specific for human VEGF, 5′-GAGAATT CGGCCTCCGAAACCATGAACTTTCTGT-3′ (sense) and 5′-GAGCATGC CCTCCTGCCCGGCTCACCGC-3′ (antisense). PCR was performed in 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U of *Taq* DNA polymerase, and 0.1 mM of each primer for VEGF. The reaction mixture was heated at 94 °C for 5 min, annealed at 62 °C for 30 s, and extended at 72 °C for 3 s for 35 repetitive cycles.

NO measurement. The level of cellular NO production was measured *in situ* by using 4-amino-5-methylamino-27-difluorofluorescein (DAF-FM) diacetate (Molecular Probes, Eugene, OR), as described previously [12]. Briefly, after treatment with sesamin (30 μM) for 1 h, the cells were washed twice with serum-free M199 and then incubated with 5 μM DAF-FM diacetate for 1 h at 37 °C. After removing the excess probe, the relative levels of intracellular NO were determined from the fluorescence intensity of DAF-FM using a confocal microscope.

[^{14}C]Sucrose permeability assay. HUVECs were plated onto a Transwell filter. After reaching confluence, HUVECs were incubated with M199 containing 1% FBS for 3 h and treated with 30 μM sesamin or 20 ng/ml VEGF for 1 h. Fifty microliters of [^{14}C]sucrose (0.8 μCi/ml; Amersham Pharmacia) was added to the upper compartment. The amount of radioactivity that diffused into the lower compartment was determined after 30 min by a liquid scintillation counter [14].

Miles vascular permeability assay. Miles assay was performed as described previously [14]. Evans blue dye (100 μl of a 1% solution in 0.9% NaCl) was injected into the tail vein of nude mice. After 10 min, 10 μl of sesamin (1 nmol/10 μl) or VEGF (45 ng/10 μl) was injected intradermally into mice. After 20 min, the animals were euthanized, and an area of skin that included the blue spot resulting from leakage of the dye was removed. Evans blue dye was extracted from the skin by incubation with formamide for 4 days at room temperature, and the absorbance of the extracted dye was measured at 620 nm with a spectrophotometer.

Statistical analysis. All data are presented as the means ± SD of at least three independent experiments. Statistical comparisons between two groups were analyzed using Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Sesamin promotes angiogenesis in vitro and in vivo

We first examined whether sesamin would promote *in vitro* angiogenesis, which requires the precise coordination of multiple processes including endothelial cell proliferation, migration, and tube formation. When HUVECs were incubated with various concentrations of sesamin, cell proliferation was significantly increased in a dose-dependent manner at concentrations between 5 and 30 μM, with a 1.5-fold increase at 30 μM, as determined by MTT assay (Fig. 1A). The effect of sesamin on endothelial cell migration was also determined by using a Transwell assay. Treatment with sesamin increased HUVEC migration in a dose-dependent manner and resulted in a 1.6-fold increase in migratory activity with 30 μM sesamin as compared with control (Fig. 1B). We next determined tube formation, following stimulation of HUVECs with various concentrations of sesamin in Matrigel-plates. Sesamin treatment resulted in a dose-dependent increase in tube-like structure formation (Fig. 1C and D). These angiogenic activities