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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

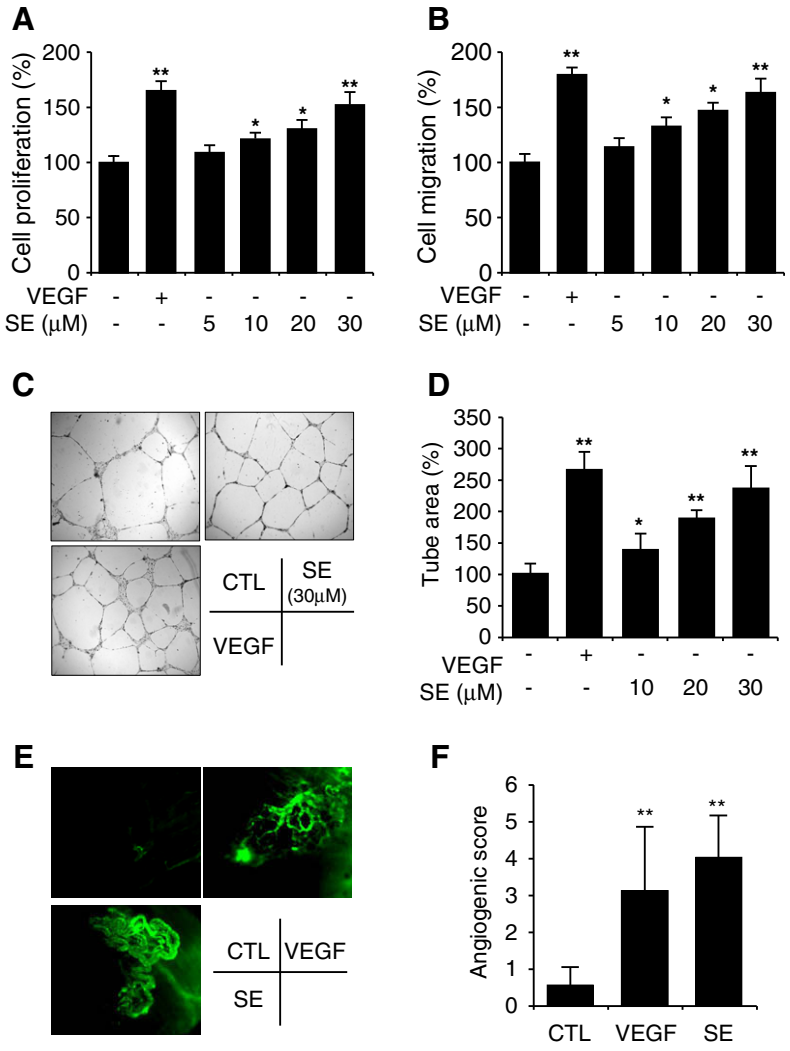

Fig. 1. Sesamin stimulates angiogenesis *in vitro* and *in vivo*. (A) HUVECs were stimulated with the indicated concentrations of sesamin (SE) or VEGF (10 ng/ml) for 24 h, and cell proliferation was determined by MTT assay. (B) HUVEC suspensions were pretreated for 30 min with the indicated concentrations of sesamin (SE) or VEGF (10 ng/ml) and loaded into the upper wells of Transwell plates. After 4 h, cell migration was quantified by counting the cells that migrated to the lower side of the filter with optical microscopy at 200× magnification. (C) HUVECs were cultured on a layer of Matrigel in the presence or absence of sesamin (SE) or VEGF (10 ng/ml) for 36 h. Tube formation was determined using a phase contrast microscope. (D) The area covered by the tube network was quantitated using Image-Pro Plus software. (E,F) Matrigel (100 μl) containing sesamin (SE, 20 nmol) or VEGF (100 ng) was injected into the abdominal window of mice, which was surgically prepared. After 4 days, neovascularization was recorded by intravital microscopy and analyzed using the MetaMorph program. Photographs were representative of *in vitro* angiogenesis. All graphical data shown are the means ± SD ($n \geqslant 3$ in cell culture experiments and $n \geqslant 6$ in animal experiments). $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* control.

of sesamin at 30 μM were relatively comparable to those of VEGF (10 ng/ml), which is a well-known pro-angiogenic stimulant (Fig. 1A–D). We further determined whether sesamin would stimulate angiogenesis in a mouse model using a fluorescence-based intravital microscopic method. Injection of Matrigel (100 μl) containing of sesamin (20 nmol) resulted in a significant increase in neovascularization compared with control, and its *in vivo* effect was also comparable to that of VEGF (100 ng) (Fig. 1E and F).

Sesamin activates ERK, Akt, eNOS, p125^{FAK}, and p38 MAPK, but not Src

It has been well demonstrated that various signal mediators such as ERK, Akt, eNOS, Src, and p38 are involved in the angiogenic signal pathway [12,13,15]. We determined whether sesamin would activate these signal mediators in sesamin-stimulated endothelial cells. Sesamin treatment resulted in significant increases in the activation of ERK, Akt, eNOS, p38 MAPK, and p125^{FAK}, but not Src, in time- and dose-dependent manners in HUVECs stimulated with sesamin (Fig. 2A–D). Since phosphorylation-dependent activation of eNOS at serine 1177 increases endogenous production of NO, which is a important modulator of angiogenesis [12], we next measured intracellular NO production as measured by the intensity of fluorescent DAF-FM formed by reaction with NO. Treatment with sesamin significantly increased NO production in a dose-dependent manner, and this increase was inhibited to basal level by the addition of the NOS inhibitor NMA (Fig. 2E and F), indicating that sesamin-induced NO production was due to eNOS activation. These results suggest that multiple signal mediators are functionally involved in sesamin-induced angiogenesis.

Sesamin-induced angiogenesis requires the ERK-, Akt/eNOS-, and p38 MAPK-dependent pathways

To investigate the functional involvement of ERK-, Akt/eNOS-, and p38 MAPK-dependent pathways in sesamin-induced angiogenesis, we determined the effects of sesamin on angiogenic processes following co-treatment with various chemical inhibitors

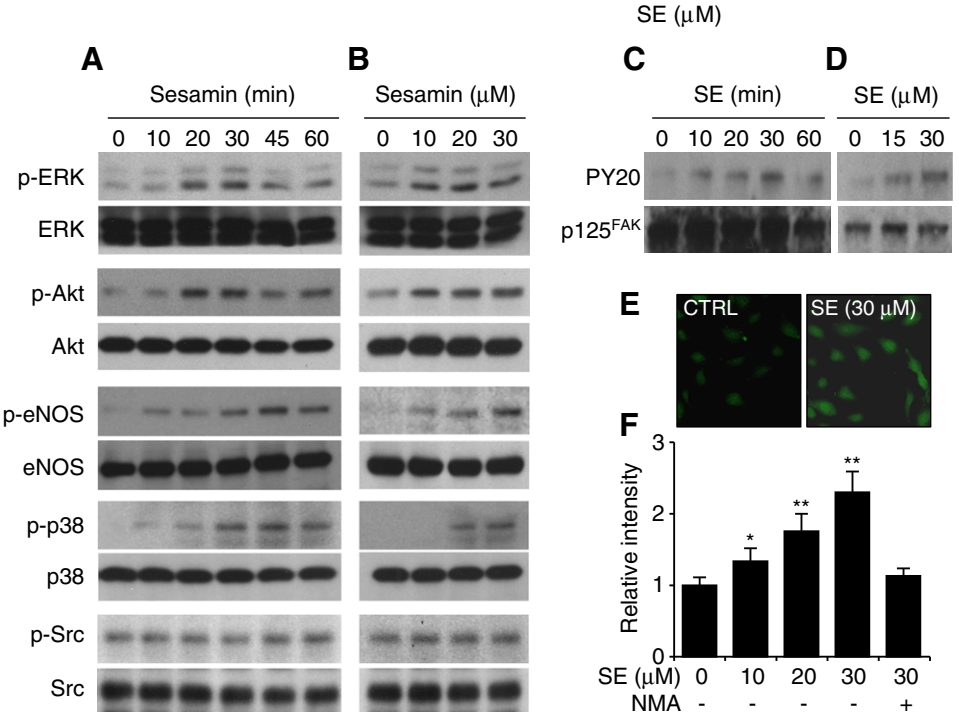

Fig. 2. Sesamin increases the activation of ERK, Akt, eNOS, and p38 MAPK, as well as intracellular NO production. (A) HUVECs were treated with sesamin (30 μM) for the indicated time periods, and the phosphorylated levels of ERK, Akt, eNOS, p38 MAPK, and Src were determined by Western blot analysis using their specific antibodies. (B) Cells were treated with the indicated concentrations of sesamin for 30 min, and the phosphorylated levels of the signal mediators were determined by Western blot analysis using their specific antibodies. HUVECs were pretreated for 30 min with 30 μM of sesamin (SE) for the indicated time periods (C) or the indicated concentrations for 40 min (D). Cell lysates were immunoprecipitated with anti-p125^{FAK} antibody, and immunoprecipitates were analyzed by SDS–PAGE. Immunoblot analysis was performed with anti-PY20. (E,F) Cells were incubated with the indicated concentrations of sesamin (SE) in the presence or absence of 1 mM NMA for 1 h, followed by incubation with DAF-FM/DA (5 μM) for 1 h. (E) The level of NO was determined with a confocal microscope. (F) The relative levels of NO were quantitated from the fluorescence intensity.

such as the MEK (upstream activator of ERK) inhibitor PD98059, the PI3K (upstream activator of Akt) inhibitor Wortmannin, the NOS inhibitor NMA, and the p38 MAPK inhibitor SB203580. Sesamin-induced phosphorylation of ERK was inhibited by PD98059, but not by Wortmannin, while Wortmannin, but not PD98059, inhibited sesamin-induced phosphorylation of Akt and eNOS (Fig. 3A). However, co-treatment with NAM was not shown to inhibit sesamin-induced activation of all these signal mediators. These inhibitors suppressed sesamin-induced endothelial cell proliferation and migration, and the inhibitory effect of PD98059 was significantly effective compared with Wortmannin and NMA (Fig. 3B and C). These inhibitors also decreased sesamin-induced tube formation of endothelial cells, and the suppressive effects of Wortmannin and NMA were higher than that of PD98059 (Fig. 3D). In addition, co-treatment with SB203580 inhibited sesamin-induced endothelial cell migration (Fig. 3E), but did not significantly suppress sesamin-induced endothelial cell proliferation (data not shown). These results suggest that sesamin-induced angiogenesis requires the activation of multiple signal cascades.

Sesamin does not induce VEGF expression, adhesion molecule expression, and hyperpermeability

Since some pro-angiogenic factors including TNF-α stimulate angiogenesis via the induction of VEGF a strong angiogenic factor [12,16], we next examined whether sesamin regulates the induction of VEGF expression. Treatment with a neutralizing antibody for VEGF did not alter sesamin-induced HUVEC proliferation, while this antibody effectively blocked VEGF-induced endothelial cell proliferation (Fig. 4A), indicating that angiogenic activity of sesamin is not associated with VEGF expression. To directly confirm this fact, we determined the level of VEGF mRNA in sesamin-stimulated HUVECs. Treatment with TNF-α, an indirect angiogenic cytokine, resulted in a significant increase in VEGF expression, whereas sesamin treatment did not increase VEGF mRNA level (Fig. 4B). We next examined whether sesamin upregulates vascular inflammation-associated adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells. Sesamin did not increase the expression of ICAM-1 and VCAM-1 in HUVECs, whereas VEGF promoted the upregulation of these adhesion molecules (Fig. 4C). We next examined whether sesamin would induce vascular hyperpermeability by transendothelial sucrose permeability in a HUVEC monolayer and a modified Miles vascular permeability in nude mice. Sesamin treatment did not increase [^{14}C]sucrose diffusion in HUVEC monolayer cultures, while VEGF significantly elevated transendothelial permeability (Fig. 4D). Moreover, spectrophotometric assay revealed that sesamin treatment did not significantly elevate vascular permeability *in vivo* compared with that induced by VEGF (Fig. 4E). These results suggest that sesamin directly stimulates angiogenesis without increasing vascular adhesion molecule expression and vascular permeability.

Discussion

This study was undertaken to elucidate the biological effect of sesamin on angiogenesis and its signal pathway in cultured HUVECs and an animal model. We showed that sesamin significantly stimulated *in vitro* and *in vivo* angiogenesis. The molecular mechanism by which sesamin promotes angiogenesis appears to involve the activation of the Akt/eNOS-, MEK/ERK, and p38 MAPK-dependent signal pathways; however, its angiogenic activity was not

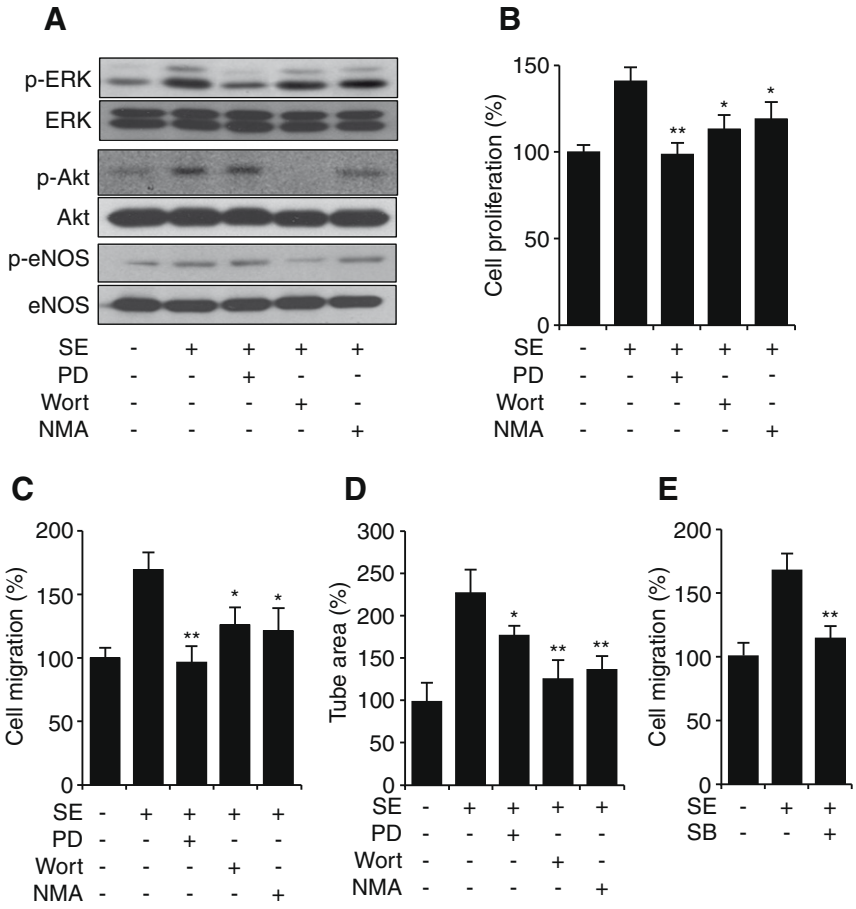

Fig. 3. Inhibitors of ERK-, Akt/eNOS-, and p38 MAPK block sesamin-induced angiogenesis. (A) HUVECs were treated with sesamin (SE, 30 μM) for 30 min following pretreatment with PD98059 (PD, 10 μM), Wortmannin (Wort, 100 nM), and NMA (1 mM) for 30 min. The phosphorylated levels of ERK, Akt, and eNOS were determined by Western blot analysis using their specific antibodies. (A–E) HUVECs were treated with sesamin (SE, 30 μM) in the presence or absence of PD98059 (PD, 10 μM), Wortmannin (Wort, 100 nM), NMA (1 mM), and SB203580 (SB, 10 μM). Cell proliferation (B), migration (C,E), and tube formation (D) were determined by the same methods as described in Fig. 1. All graphic data are the means ± SD ($n \geqslant 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* sesamin alone.

associated with VEGF expression. In addition, this compound did not induce the expression of ICAM-1 and VCAM-1, which are typical indicators of vascular inflammation. These findings suggest that sesamin may have therapeutic implications for diseases characterized by ischemic injury and damage.

It has been shown that angiogenic factors increase neovascularization via two distinct action modes such as direct activation of the angiogenic signal pathways and production of angiogenic factors including VEGF [17]. Direct angiogenic factors including VEGF promote angiogenesis by activating multiple signal pathways, such as the MEK/ERK pathway for endothelial cell proliferation, the PI3K/Akt/eNOS pathway for endothelial cell survival, and the $p125^{FAK}$/Src/p38 MAPK signaling system for endothelial cell migration [12,17]. It has been shown that VEGF-induced endothelial cell migration requires the concerted activation of two independent mechanisms leading to stress fiber formation: one involves p38 MAPK-mediated actin polymerization and the other implicates $p125^{FAK}$-dependent proper assembly of focal adhesions [18]. On the other hand, some other angiogenic inducers including TNF-α and CXCL8/IL8 indirectly evoke angiogenesis via the upregulation of VEGF expression [16,19,20]. Our data showed that sesamin increases angiogenesis without the induction of VEGF expression, indicating that this compound may directly activate the angiogenic signal pathways. Indeed, we confirmed that sesamin elicited the activation of multiple angiogenic signal cascades, such as MEK/ERK-, PI3K/Akt/eNOS-, $p125^{FAK}$-, and p38 MAPK-dependent pathways, which are essential for the activation of endothelial cells induced by direct angiogenic factors [17]. However, our results also demonstrated that sesamin-induced endothelial cell migration is not associated with Src activation. These results indicate that sesamin is a direct angiogenic inducer, which activates intracellular angiogenic signal mediators without elevating VEGF expression.

Angiogenic proteins including VEGF and placental growth factor have been used to treat ischemic diseases and peripheral artery disease through the improvement of blood flow by increasing angiogenesis. It is generally accepted that protein drugs have some limitations such as low stability and short half-life. In addition, the potential angiogenic inducer VEGF has been shown to cause some considerable adverse effects, such as vascular inflammation and vascular permeability [21,22]. Therefore, there is a great interest in developing more stable small molecules that can potentially induce angiogenesis, without these harmful effects, for therapeutic application to ischemic vascular diseases. Our study showed that sesamin significantly stimulated *in vitro* and *in vivo* angiogenesis without the induction of the vascular inflammatory molecules (ICAM-1 and VCAM-1) and elevation of vascular permeability, indicating that this compound will give the pharmacological effect and activity for therapeutic angiogenesis.

In conclusion, out study demonstrated that sesamin stimulated *in vitro* and *in vivo* angiogenesis, which was followed by the activation of multiple angiogenic signal cascades, such as the MEK/ERK-, PI3K/Akt/eNOS-, $p125^{FAK}$-, and p38 MAPK-dependent pathways,

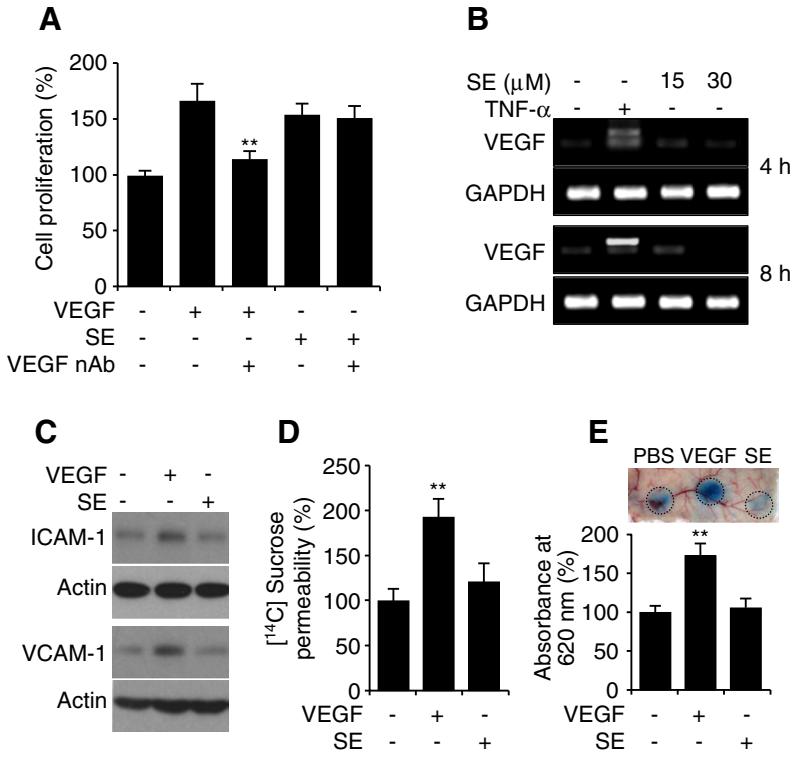

Fig. 4. Sesamin does not affect the expression of VEGF and adhesion molecules as well as transendothelial permeability. (A,B) HUVECs were treated with sesamin (SE, 30 μM), VEGF (10 ng/ml), or human TNF-α (10 ng/ml) following pretreatment with a neutralizing VEGF antibody (1 μg/ml, VEGF nAb) for 10 min. (A) After 24 h, cell proliferation was determined by MTT assay. Data are the means ± SD ($n \geqslant 3$). $^{**}p < 0.01$ *versus* VEGF. (B) The level of VEGF mRNA was determined by RT-PCR analysis. TNF-α was used as a positive control. (C) Cells were treated with treated with sesamin (SE, 30 μM) or VEGF (10 ng/ml) for 6 h. The protein levels of ICAM-1 and VCAM-1 were determined by Western blot analysis. (D) Cells cultured onto a Transwell filter were treated with sesamin (SE, 30 μM) or VEGF (20 ng/ml) for 1 h and then incubated with [^{14}C]sucrose for 30 min. The amount of radioactivity that diffused into the lower compartment was determined by a liquid scintillation counter. Data are the means ± SD ($n = 3$). $^{**}p < 0.01$ *versus* untreated control. (E) Ten microliters of sesamin (SE, 1 nmol/10 μl) or VEGF (45 ng/10 μl) was injected intradermally into the skin of nude mice following intravenous injection of Evans blue. After 20 min, Miles vascular permeability was assayed. Representative picture (top) and quantity (bottom) of extravasated Evans blue in the mouse skin. Data are the means ± SD ($n = 6$). $^{**}p < 0.01$ *versus* untreated control.

without inducing VEGF expression. In addition, this compound is a potential direct angiogenic inducer without vascular inflammation and vascular permeability. Although further therapeutic study is needed in an ischemic animal model, sesamin should be considered a potential candidate as a therapeutic drug for ischemic diseases, which are required for the restoration of blood flow via angiogenesis.

Acknowledgments

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