Isoliquiritigenin inhibits VEGF-mediated angiogenesis by regulating the distinct signalling of VEGFR-1 and VEGFR-2 via MAPKs activation in HUVECs

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Isoliquiritigenin inhibits VEGF-mediated angiogenesis by regulating the distinct signalling of VEGFR-1 and VEGFR-2 via MAPKs activation in HUVECs

Directed by Professor Kwang-Kyun Park

The Master's Thesis submitted to the Department of Dentistry, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Dentistry

Seung Hwa Son

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This certifies that the Master's Thesis

of Seung Hwa Son is approved.

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어. 알지? 더 열심히 할께.”고마워 윤정아 - 사랑합니다.

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승화를 너무나 아껴주시는 장만석 선생님, 내가 자랑스럽다고 말해준 근영이 엄마-성민, 점점 예뻐지는 학구파-선, 결혼은 진정 행복이던가? 부럽다-혜영&기현, 지금은 함께 있지 못하는 천상 귀염둥이-군하, 십장생 후배들-지현,주미,미수,효은,미경, 선희,혜영,혜정,은미, 일본에서 힘들게 공부하고 있을 멋진 친구-상호, 건빵에는 별사탕이 필요하다는 걸 가르쳐 준 친구-승후, 선인장 같은 필재오빠와 인생선배 재균오빠에게 고마움을 전합니다. 마지막으로 - 내 평생의 친구이자 세상에 하나뿐인 동생 윤정이에게 이 논문을 바칩니다.

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Isoliquiritigenin inhibits VEGF-mediated angiogenesis by regulating the distinct signalling of VEGFR-1 and VEGFR-2 via MAPKs activation in HUVECs

Seung Hwa Son

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(Directed by Professor Kwang-Kyun Park)

During tumor growth, angiogenesis - the formation of new capillaries from preexisting ones - is required for proper nourishment and removal of metabolic wastes from tumor sites. Angiogenesis is the result of an intricate balance between pro-angiogenic and anti-angiogenic factors and is now very well recognized as a powerful control point in tumor development. A number of pro-angiogenic factors have been identified, the most potent of which is vascular endothelial growth factor (VEGF)/ vascular permeability factor (VPF). VEGF is secreted by many tumors and initiates essential steps of angiogenesis including matrix degradation and endothelial cell proliferation and migration. VEGF is an intensively studied molecule that has significant potential, both in stimulating angiogenesis and as a target for antiangiogenic approaches. VEGF achieves its multiple functions by activating two receptor tyrosine kinases, VEGF Receptor-1 (VEGFR-1/ Flt-1) and VEGF Receptor-2

(VEGFR-2/ KDR), both of which are selectively expressed on primary vascular endothelium. Therefore, blocking the binding of VEGF and the corresponding receptor has become critical for anti-angiogenesis therapy.

In the current study, we found that isoliquiritigenin (ISL) inhibited VEGF-induced cell proliferation, DNA synthesis, cell migration and tube formation in human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner. Antiangiogenic activity of ISL was confirmed by in vivo Matrigel plug assay. Moreover, to explain the molecular mechanism underlying its antiangiogenic activity, we examined the effects on the distinct signaling of VEGFR-1 and VEGFR-2 closely associated with the proliferation and migration of VEGF-stimulated HUVECs. ISL reduced not only the expression of VEGFR-1, VEGFR-2, focal adhesion kinase (FAK) and paxillin but also the phosphorylation of MAPKs, FAK and paxillin, in VEGF-treated HUVECs. VEGFR-2-mediated activation of ERK1/ 2 and JNK increased cell proliferation and migration through the phosphorylation of FAK and paxillin in VEGF-treated HUVECs. VEGFR-2 also stimulated the activation of p38 MAP kinase which is responsible for actin reorganization and subsequent cell migration. In addition, VEGF may induce cell proliferation and migration by VEGFR-1-mediated JNK and p38 MAP kinase activation. Taken together, ISL has the potent antiangiogenic activity by blocking VEGFR signaling in VEGF-stimulated endothelial cells.

Key words : isoliquiritigenin, angiogenesis, VEGF, VEGFR-1, VEGFR-2, HUVEC

Ⅰ. INTRODUCTION

Angiogenesis, the formation of new vessels from preexisting ones, is a strictly regulated and self-restricted physiological process. A growing number of diseases, including rheumatic arthritis, psoriasis, and diabetic retinopathy, derive from excessive and deregulated angiogenesis. However, the most important manifestation of pathological angiogenesis is that seen in the vicinity of solid tumors[1]. One of the most important factors regulating angiogenesis is vascular endothelial growth factor (VEGF). Indeed, most tumors express high levels of VEGF. VEGF expression in breast cancer is well documented and VEGF is produced by both macrophages and cancer cells in breast carcinoma[23,62]. VEGF, the angiogenic factor most commonly produced by tumors, is overexpressed after hypoxia[3,4] and/ or as a consequence of the genetic changes of cancer, such as mutations of oncogenes and tumor suppressor genes[5]. The adjacent ECs express VEGF receptor 1 and 2[6], establishing an angiogenic loop (Fig. 1). VEGF regulates key angiogenic responses of endothelial cells (ECs), for instance proliferation, migration, and differentiation, as well as protection from apoptosis[2].

VEGF binds to two receptor-type tyrosine kinases, VEGF receptor-1 (Flt-1) and VEGF receptor-2 (KDR/ Flk-1), and to membrane protein neuropilin-1, which does not contain a tyrosine kinase domain. VEGFR-1 and VEGFR-2 have seven immunoglobulin-like repeats in the extracellular domain, a single trans-membrane region, and a tyrosine kinase domain in the intracellular domain that is interrupted by a kinase-insert domain. VEGFR-2 appears to play a critical role in the regulation of angiogenesis[7], whereas VEGFR-1 also seems to participate in pathological angiogenesis[8]. VEGFR-2 has tyrosine kinase activity and phosphorylates secondary messengers, which appear to regulate EC proliferation via activation of extracellular signal-regulated kinase (ERK1/ 2) mitogen activated protein kinases (MAPKs)[9], migration of ECs via the stress-activated protein kinase 2/ p38 pathway[10], and

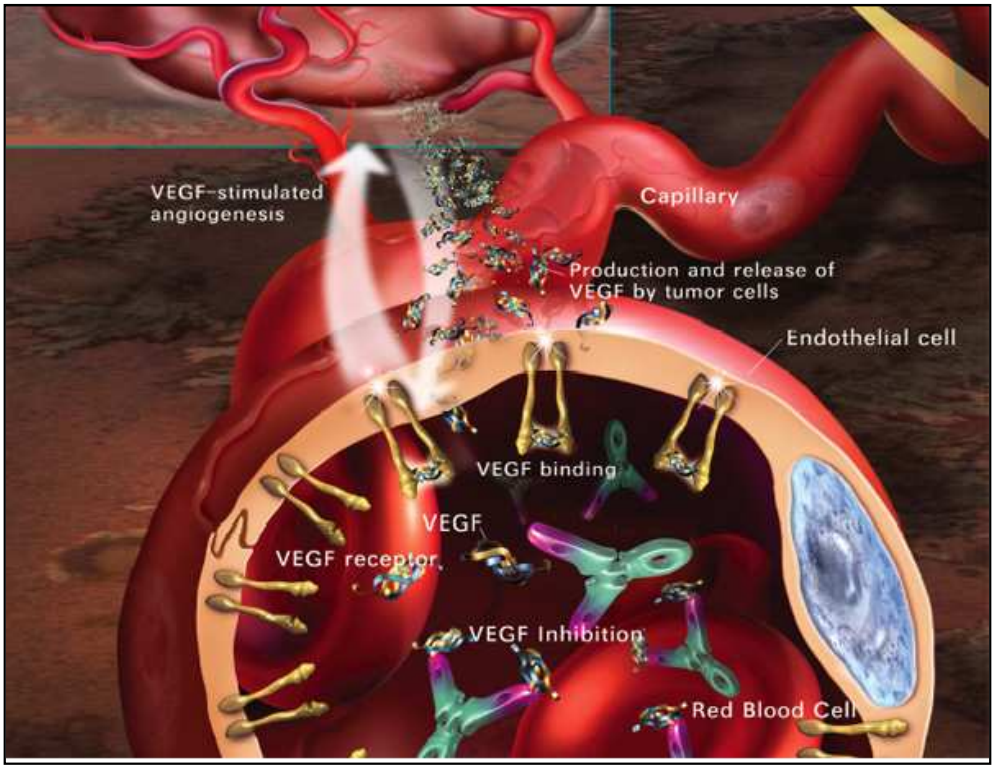


Figure 1. Angiogenesis is driven by the tumor's release of pro-angiogenic signals, such as VEGF, which bind to receptors on nearby vessel endothelial cells. (Genentech BioOncology™ "Signaling Pathways" poster)

survival of ECs via Akt activation[11]. Activation of the VEGFR-2 by VEGF in cells devoid of VEGFR-1 results in a mitogenic response, while the activation of VEGFR-1 by VEGF in cells lacking VEGFR-2 does not induce cell proliferation[12,13]. However, activation of VEGFR-1 by VEGF does induce cell migration, a response that is also induced as a result of VEGFR-2 activation by VEGF[14,15]. These results indicate that the signal transduction cascades induced by VEGFR-1 and VEGFR-2 are somewhat different. In spite of these observations, properties of VEGFR-1 and VEGFR-2 in normally differentiated ECs are controversial.

Focal adhesion kinase (FAK) is a critical mediator of signal transduction by integrins and growth factor receptors in a variety of cells including ECs. FAK binding to Src family kinases contributes to the activation of both kinases, which leads to phosphorylation of several other sites on FAK and a number of other substrates including paxillin, and Shc. FAK and its interactions with these signaling molecules have been shown to trigger several downstream signaling pathways that regulate cell spreading and migration, cell survival, and cell cycle progression. In addition, increased EC migration into a wounded monolayer was correlated with increased tyrosine phosphorylation and kinase activity of FAK[22].

VEGF induces tyrosine phosphorylation of FAK and paxillin[24] and requires FAK to promote new focal adhesion formation in human umbilical vein endothelial cells (HUVECs)[25]. FAK activation is mediated via the C-terminal tail of VEGFR-2 and loss of VEGF-induced FAK activation in cells expressing mutant VEGFR-2 correlates with a loss of migratory activity regulated through PI3 kinase[24]. Via another independent pathway, VEGF activates p38 MAP kinase in HUVECs. VEGF-induced actin reorganization and cell migration is inhibited using a specific p38 kinase inhibitor, whereas an ERK1/ 2 MAP kinase inhibitor has no effect on this cellular response[10,27]. In addition, a novel rapid cross-activation of c-Jun N-terminal kinase (JNK) by ERK is crucial to the ability of VEGF and probably other EC growth factors to enact cell proliferation, contributing to angiogenesis[28]. VEGFR-2 mediates the signals for DNA

synthesis, phosphorylation of FAK and paxillin, and vinculin assembly. The signals for DNA synthesis are mediated via PKC-dependent and ERK1/2-dependent pathway, whereas those for phosphorylation of FAK and paxillin and vinculin assembly are preferentially mediated via PKC-dependent and ERK1/2-independent pathway. VEGFR-1 activates p38 MAP kinase, and the activated p38 regulates cell migration via actin reorganization[29].

Isoliquiritigenin (ISL), a flavonoid with a chalcone structure, has been found in licorice, shallot and bean sprouts and often used in Chinese medicine. It has various biochemical activities such as antioxidative and superoxide scavenging activities[33], antiplatelet aggregation effect[34], inhibitory effect on aldose reductase activity[35], and estrogenic properties[32]. In addition, ISL was reported to have antitumor promoting activity[31], inhibitory effect on murine colonic tumorigenesis[38], and apoptosis-inducing activity[30] even though stimulatory effects on T-47D breast cancer cells have been documented[32].

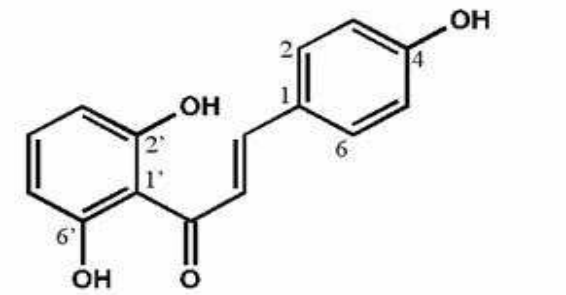
In the present study, we assessed the inhibitory effect of ISL on VEGF-induced angiogenesis in HUVECs. Moreover, we investigated the effect of ISL on several signaling pathways emanating from VEGFR-1 and/or VEGFR-2 leading to MAP kinase activation in response to VEGF in HUVECs.

Ⅱ. MATERIALS AND METHODS

1. Materials and antibodies

Isoliquiritigenin (ISL) [(E)-1-(2,4-Dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one; 4,2′4′-trihydroxychalcone] (Fig. 2) was purchased from Sigma (St. Louis, MO, USA) and was used after dilution in DMSO. MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide] and DMSO (dimethylsulfoxide) were purchased from Sigma (St. Louis, MO, USA). Recombinant human vascular endothelial cell growth factor (VEGF ; 10 ㎍/ 50 ㎕) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). SB203580 [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole], PD98059 [2′-Amino-3′-methoxyflavone], SP600125 [Anthra[1,9-*cd*]pyrazol-6(2*H*)-one; 1,9-pyrazoloanthrone] and ZM323881 [5-((7-(Benzyloxy)quinazolin-4-yl)amino)-4-fluoro-2-methylphenol; KDR/ Flk-1 inhibitor] were purchased from Calbiochem (La Jolla, CA, USA). Affinity-purified polyclonal antibidies of paxillin, p-paxillin and Flt-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The following antibodies were used for Western blotting : SAPK/ JNK, p-SAPK/ JNK Thr183/ Tyr185 and ERK1/ 2 (Cell Signaling Technology, Danvers, MA, USA) and FAK and FAK pY397 (BD Biosciences, San Diego, CA, USA). Monoclonal antibodies (MAbs) of p38, p-p38 and p-ERK1/ 2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Flt-1 was purchased from R&D Systems (Minneapolis, MN, USA), and anti-KDR was obtained from R&D Systems (Minneapolis, MN, USA) and Abcam Ltd. (Cambridge, United Kingdom). Rabbit anti-actin as a standard control and heparin were obtained from Sigma (St. Louis, MO, USA). Horseradish peroxides-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2. Cell culture



Licorice root **Isoliquiritigenin (4, 2', 4'-trihydroxychalcone)**

Figure 2. **Licorice root and the structure of isoliquiritigenin**

HUVECs were isolated by collagenase digestion of umbilical veins from undamaged sections of fresh cords. The cells were maintained in EBM-2 medium (Cambrex Bio Science, Walkersville, MD, USA) containing 20% FBS. Sub-cultures were obtained by trypsination and were used at passages > 6 in this study. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

3. MTT assay

The effects of ISL or MAPKs inhibitors on the viability of VEGF-treated HUVECs were evaluated by MTT assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. HUVECs were plated at a density of $5X10^3$ cells/ well into a 96-well culture plate (NUNC, Roskilde, Denmark) and left overnight to adhere. Then, ISL at various concentrations or MAPKs inhibitors (10 μM) was added to the medium in the presence or absence of VEGF (20 ng/ ml) and the cells were incubated for 24 and 72 hr. After the MTT addition (5 mg/ ml), the plates were incubated for 4 hr at 37 °C in 5% CO_2 incubator. The supernatant was carefully removed from the wells, formazan product was dissolved in 200 ㎕ DMSO, and absorbance was measured at 570 nm in a microplate reader (BIO-RAD, Hercules, CA, USA). The OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells.

4. 5-bromo-2'-deoxyuridine (BrdU) incorporation assay

DNA synthesis was measured by BrdU labeling method with commercially available kits (Roche Diagnostics, Mannheim, Germany). Briefly, HUVECs ($5X10^3$ cells/ well) were seeded in EBM-2 medium containing 20% FBS in 96-well culture plate and incubated overnight at 37 °C, 5% CO_2. Various concentrations of ISL or MAPKs inhibitors (10 μM) in serum-free medium containing VEGF (20 ng/ ml)

were added to the each well. Cells were incubated for 24 and 72 hr and BrdU (final concentration : 10 μM/ well) was added. After 4 hr incubation at 37 °C, medium was removed and cells were fixed with ethanol (70%) in HCl (final concentration : 0.5 M) for 30 min at -20 °C. Cells were incubated with nucleases working solution for 30 min at 37 °C in the absence of CO_2 and incubated with a solution containing an anti-BrdU-POD, Fab fragments, for 30 min at 37 °C. After 30 min unbound antibody was removed by 3 times washings. POD substrate solution (3,3′-5,5′-tetramethylbenzidine, 100 ㎕ per well) was added and incubated at room temperature until positive samples show a green color, and is clearly distinguishable from the color of pure POD substrate. Absorbance of the samples was immediately measured in a microplate reader at 405 nm (reference wave-length at approx. 490 nm).

5. Cell migration assay

The chemotactic motility of HUVECs was assayed using Transwell (Corning Costar, Cambridge, MA, USA) with 6.5 mm-diameter polycarbonate filters (8 ㎛ pore size) and 10 ㎍ of gelatin matrix in the upper chamber. In the present study, 100 ㎕ of cell suspension ($1X10^6$ cells/ ml) in EBM-2 medium (FBS 2%) was added to upper chamber, while the lower chamber contained 600 ㎕ EBM-2 medium with 2% FBS or EBM-2 with 20 ng/ ml VEGF supplemented with 5-40 μM ISL or vehicle. The apparatus was incubated for 7 hr at 37 °C, and then the upper chambers were disassembled and fixed with 70% methanol for 5 min. The membranes were stained with hematoxylin/ eosin (H.E) for 5 min. Cells remaining on the upper surface of the filter membrane (nonmigrant) were scraped off gently with a cotton swab and then mounted on slide glasses. Cell migration was quantified by counting the whole cell numbers on a single filter using an optical microscope at x 200 magnification. Assays were done in triplicate and repeated at least twice.

6. Tube formation assay

A tube formation assay was used to investigate the effect of ISL on angiogenesis in vitro. The HUVECs at subconfluence were switched to serum-free EBM-2 medium overnight. 250 $\mu\ell$ of growth factor-reduced Matrigel (Collaborative Biomedical Products, Bedford, MA, USA) was placed in a 24-well culture plate and polymerized for 30 min at 37 °C. The starved HUVECs were trypsinized and suspended in EBM-2 containing 2% FBS. The cells were mixed with various concentrations of ISL and VEGF (20 ng/ ml) and seeded to the Matrigel pretreated 24-well culture plate at a density of 1×10^5 cells/ well. After 8 hr incubation at 37 °C, the morphological changes in the cells were observed under a microscope and photographed at × 40 magnification.

7. Animals

7-week old male C57/ BL6 mice were obtained from Orient Co. (Seoul, Korea) that were maintained at 20-22 °C with 12 hr light and dark cycles.

8. Matrigel plug assay in vivo

Matrigel plug assay was performed as previously described[40]. Angiogenesis was considered by the growth of blood vessels from subcutaneous tissue into a solid gel of Matrigel extracellular matrix. The Matrigel (BD Biosciences, Palo Alto, USA) was thawed overnight at 4 °C. Various concentrations of ISL were gently mixed with cold liquid Matrigel containing the 20 units of heparin with or without 100 ng/ ml VEGF. The Matrigel solution (0.6 ml) was injected subcutaneously into flank of mice. To investigate the bioavailability of ISL, ISL (0.25 - 0.5 mg/ kg/ day) was orally given once every day after C57/ BL6 mice were given subcutaneous injections of 0.6 ml of Matrigel with VEGF (100 ng/ ml). 7 days later, the mice were killed by ethyl ether inhalation and the skin of the mouse was easily pulled away to expose the Matrigel plug. After the Matrigel plugs were surgically removed and

photographed, the amount of hemoglobin (Hb) inside the Matrigel were measured as an indication of blood vessel formation using the Drabkin's reagent kit 525 (Sigma, St. Louis, MO, USA). The concentration of Hb was calculated based on Hb standard measured simultaneously.

9. Western blot analysis

Protein lysates were prepared using lysis buffer containing 5 mM EDTA, 50 mM Tris-HCl, 0.1% SDS, 10% glycerol, 0.2% Triton X-100, 5 μg/ ml aprotinin, 1 mM PMSF and protease inhibitor cocktail tablet (Roche, Penzberg, Germany). Protein concentrations were determined using a BCA kit (Pierce, Rockford, IL, USA). Equal amounts of protein (30 μg) were loaded in each lane and separated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresed proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% skim milk in TBS-T, the membranes were incubated with primary antibodies (1:1000) in 3% skim milk overnight at 4 °C. The membranes were incubated with an appropriate peroxidase-conjugated secondary antibody (1:2000) in 3% skim milk for 1 hr at room temperature and then developed with an enhanced chemiluminescence (ECL) mixture (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

10. Statistical analysis

Data from the in vitro and in vivo experiments are expressed as the mean ± standard error (SE). Comparisons between groups were carried out by Student's *t*-test for multiple comparisons where appropriate. Differences were considered significant at the level of $P \leq 0.05$.

Ⅲ. RESULTS

ISL suppresses VEGF-induced cell viability in HUVECs

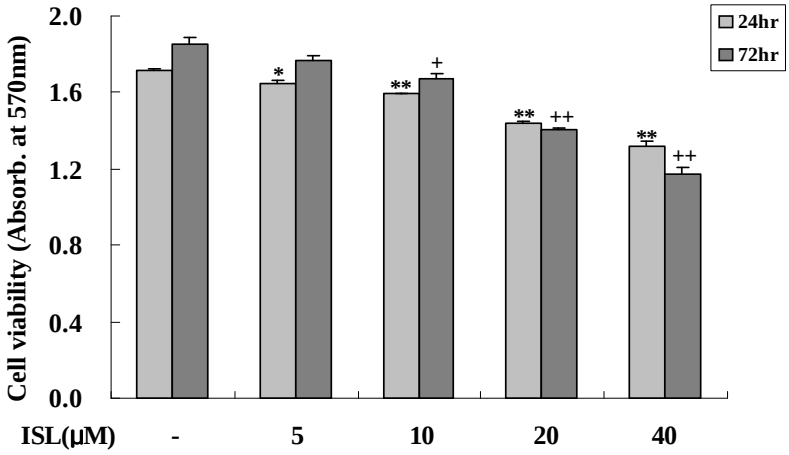
Vascular endothelial cell proliferation is an essential component of the angiogenic responses. The effects of ISL on HUVECs were investigated by the MTT assay at the concentrations of ISL ranging from 0 to 40 μM for 24 and 72 hr, respectively. ISL inhibited HUVEC proliferation in the absence (Fig. 3A) or presence (Fig. 3B) of VEGF (20 ng/ ml). To examine whether ISL was toxic to HUVECs, we treated with ISL in the absence of VEGF. The viability of HUVECs was reduced to 83% and 81% at 20 μM of ISL and remarkedly inhibited to 71% and 63% at 40 μM, respectively, when treated for 24 and 72 hr. Addition of VEGF (20 ng/ ml) caused a significant increase in the viability of HUVECs and ISL treatment decreased VEGF-induced cell viability in HUVECs in a dose-treated manner.

ISL inhibits VEGF-induced DNA synthesis of HUVECs

The cell proliferation was measured by quantitating BrdU incorporated into the newly synthesized DNA of replicating cells. When cultured HUVECs with ISL in the absence of VEGF for 24 and 72 hr, but no effect on their DNA synthesis was found (Fig. 4A). Addition of VEGF (20 ng/ ml) caused a significantly increased DNA synthesis in HUVECs and ISL treatment at 40 μM for 24 hr resulted in 70-75% ($P<0.002$) inhibition of VEGF-induced DNA synthesis (Fig. 4B). These results suggest that reduction of VEGF-induced cell viability by ISL in HUVECs is due to the inhibition of cell proliferation by blocking DNA synthesis.

ISL inhibits VEGF-induced cell migration and tube formation in HUVECs

A


Cell viability (Absorb. at 570nm)
2.0
1.6
1.2
0.8
0.4
0.0
24hr
72hr
*
**
+
**
++
**
++
ISL(μM) - 5 10 20 40

B

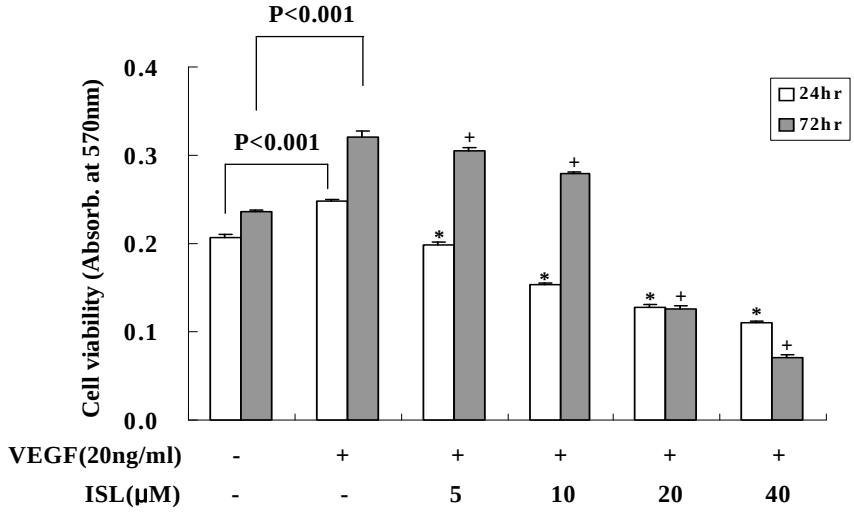
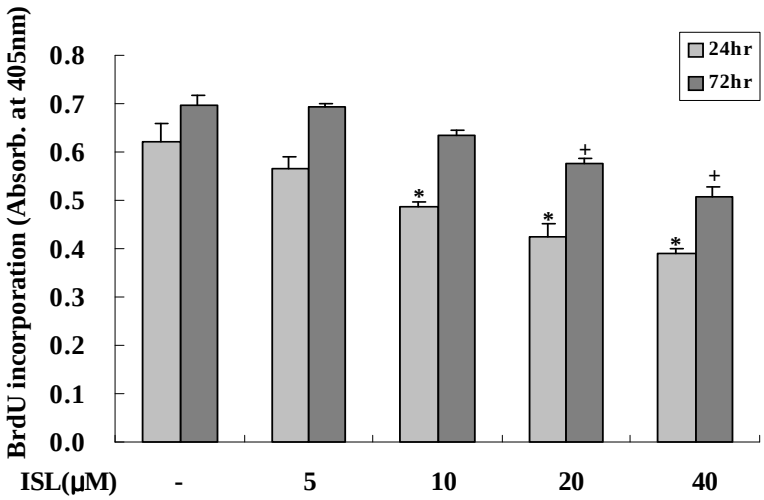

P<0.001
P<0.001
Cell viability (Absorb. at 570nm)
0.4
0.3
0.2
0.1
0.0
24hr
72hr
VEGF(20ng/ml) - + + + + +
ISL(μM) - - 5 10 20 40

Figure 3. ISL suppresses VEGF-induced viability of HUVECs. Cells were treated with or without ISL (5 - 40 μM) and VEGF (20 ng/ ml) for 24 and 72 hr, respectively, and the proliferation was measured using MTT assay. Each experiment was done independently in triplicate twice. Data are expressed as the means ± S.E. *, P<0.05 ; **, P<0.001 *versus* VEGF-treated (or untreated) group for 24 hr ; +, P<0.05 ; ++, P<0.001 *versus* VEGF-treated (or untreated) group for 72 hr.

A


BrdU incorporation (Absorb. at 405nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
24hr
72hr
ISL(μM) - 5 10 20 40

B

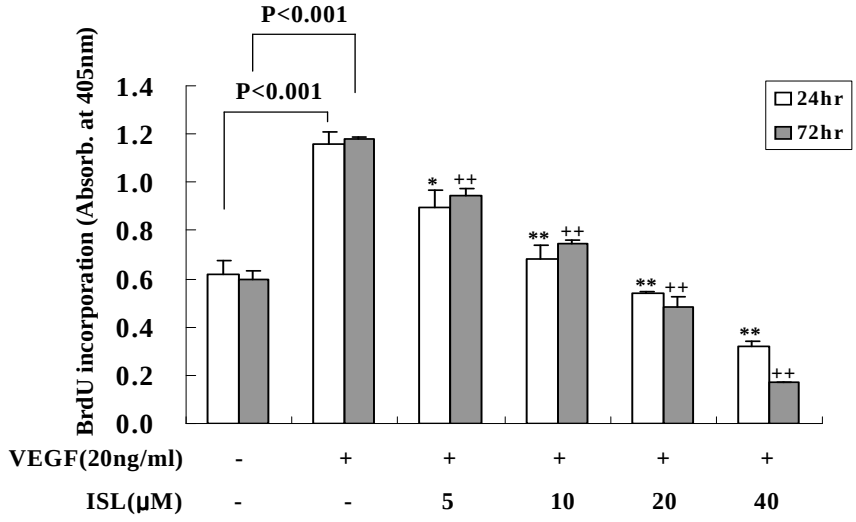

P<0.001
P<0.001
BrdU incorporation (Absorb. at 405nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
24hr
72hr
VEGF(20ng/ml) - + + + + +
ISL(μM) - - 5 10 20 40

Figure 4. ISL inhibits VEGF-induced DNA synthesis of HUVECs. In BrdU incorporation assay, $5X10^3$ cells/ well were plated in 96-well culture plate in culture conditions as described in Material and Method, and treated with or without ISL (5 - 40 μM) and VEGF (20 ng/ ml) for 24 and 72 hr, respectively. At the end of treatment, cells were incubated with BrdU labeling solution followed by fixation and incubation with anti-BrdU peroxidase conjugate. Finally, after substrate reaction, color intensity was measured with microplate reader at 405 nm. Data represent the mean ± S.E. of two independent experiments. *, P<0.05 ; **, P<0.002 *versus* VEGF-treated (or untreated) group for 24 hr ; +, P<0.01 ; ++, P<0.001 *versus* VEGF-treated (or untreated) group for 72 hr.

Angiogenesis is a complex process involving endothelial cell proliferation, migration and differentiation as well as tube formation. The effect of ISL on the chemotactic motility of HUVECs was measured using a Transwell plate. When added for 8 hr in the lower well, VEGF (20 ng/ ml) induced a strong activation of cell migration, but VEGF-stimulated cell migration was completely blocked by ISL treatment (Fig. 5).

Next, the effect of ISL on the morphological differentiation of HUVECs was investigated on two-dimensional Matrigel. When HUVECs were placed on growth factor-reduced Matrigel in the absence of VEGF, HUVECs formed incomplete and narrow tube-like structures. The treatment of 20 ng/ ml VEGF led to the tube formation of elongated and robust tube-like structures, which were organized by much larger number of cells compared with the control. However, ISL effectively abrogated the width and the length of endothelial tubes induced by VEGF in a dose-dependent manner (Fig. 6). Tube formation in the VEGF-treated HUVECs inhibited incompletely by treatment with 1 μM of ISL but remarkably inhibited by 10 μM of ISL. These results indicate that ISL inhibits VEGF-induced migration and tube formation in HUVECs.

ISL inhibits VEGF-induced angiogenesis *in vivo* Matrigel plug assay

To examine whether VEGF-induced angiogenesis could be inhibited by ISL in vivo, we conducted Matrigel plug assays in mice. Subcutaneous injection of cold liquid Matrigel containing VEGF (100 ng/ ml) permitted the formation of new blood vessels. 7 days later, Matrigel with VEGF markedly induced angiogenesis the 5-fold increases in comparison with control Matrigel alone. In contrast, blood vessel formation significantly inhibited in ISL-treated Matrigel with VEGF by 68% (Fig. 7A). We also evaluated the effect of the oral administration of ISL on VEGF-induced angiogenesis. As expected, oral administration of ISL significantly inhibited VEGF-induced

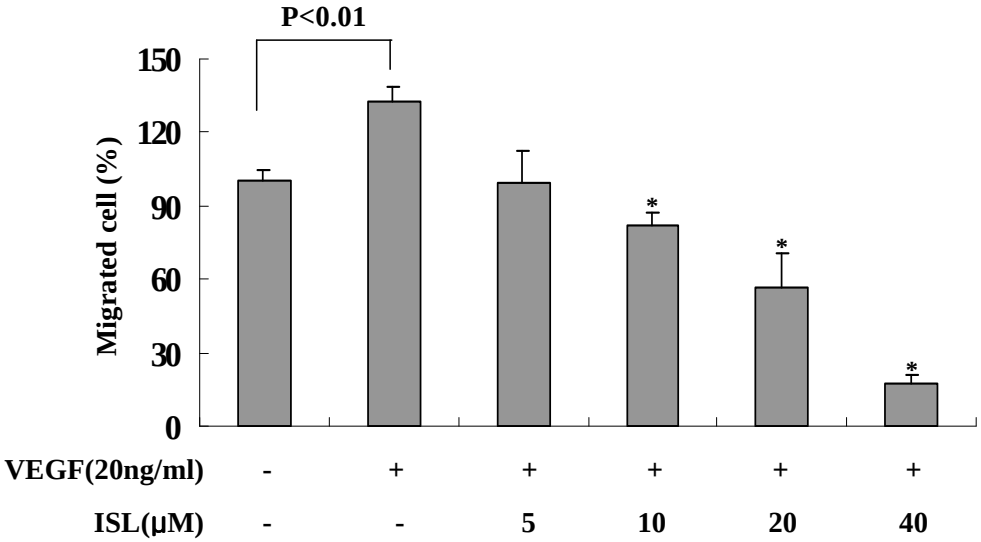


Figure 5. ISL inhibits VEGF-induced migration in HUVECs. HUVECs ($1X10^6$ cells/ ml) were seeded on top of the Transwell membrane and allowed to migrate through 8 ㎛ pores for 8 hr. Migration of HUVECs exposed to EBM-2 medium containing 2% FBS with and without ISL (5 - 40 μM) plus VEGF (20 ng/ ml) was analyzed by Transwell assay as detailed in Materials and methods. Migrated cell (%) represents the mean number of migrating cells/ field ± SE calculated in three different wells. Experiment was repeated at least twice. *, P<0.05 *versus* VEGF-treated group.

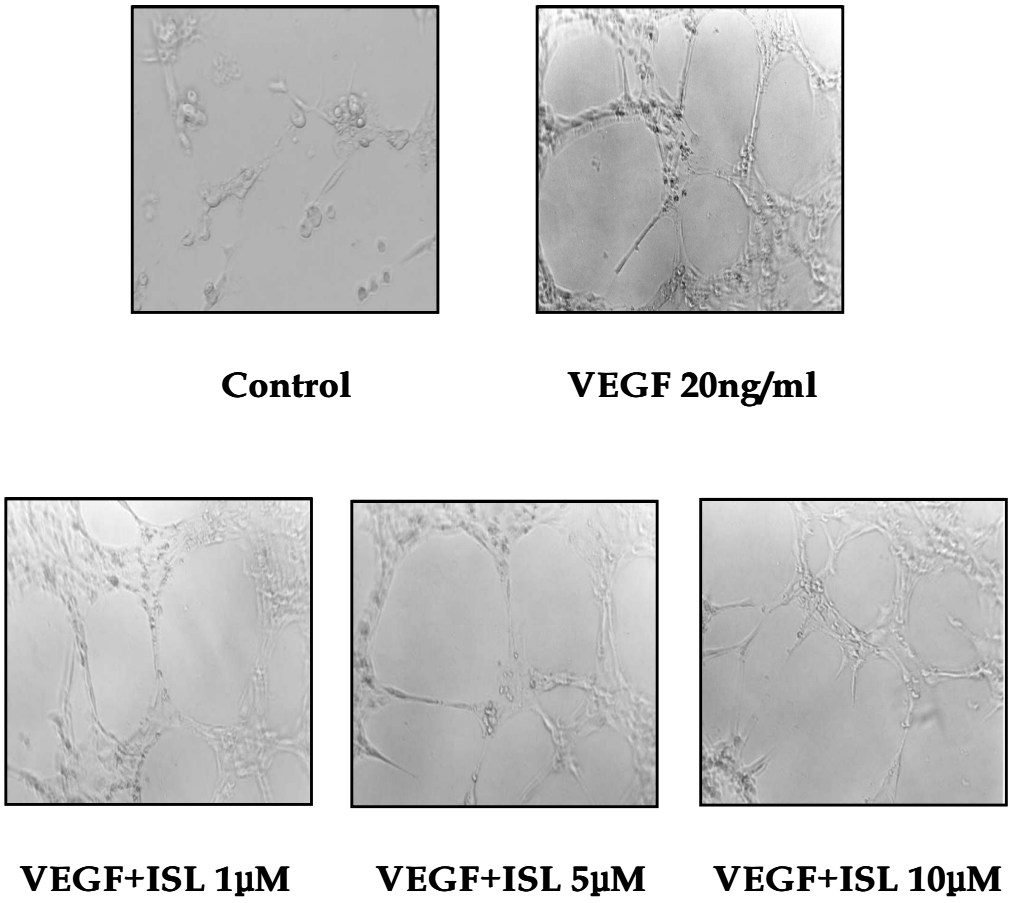


Figure 6. ISL inhibits VEGF-induced tube formation in HUVECs. HUVECs ($1X10^5$ cells/ well) were seeded into 24-well plate which had been precoated with growth factor-reduced Matrigel in EBM-2 medium containing 2% FBS. The cells were treated with various concentrations of ISL (1 - 10 µM) in the absence or presence of VEGF (20 ng/ ml) for 7 hr. Photomicrographs depict the alignment of HUVEC under defined treatment conditions.

angiogenesis as shown in Fig. 7B. Furthermore, we found that subcutaneous injection of Matrigel with ISL 20 μM (5 ㎍/ ml) decreased VEGF-induced angiogenesis more significantly than its oral administration of 0.25 mg/ kg/ day every day for 7 days (the total amount of orally administrated ISL : 5 ㎍/ ml) (Fig. 7C). These results demonstrate that ISL effectively inhibits VEGF-induced angiogenesis in vivo system.

ISL downregulates VEGF-stimulated VEGFR-1 and VEGFR-2 expression in HUVECs

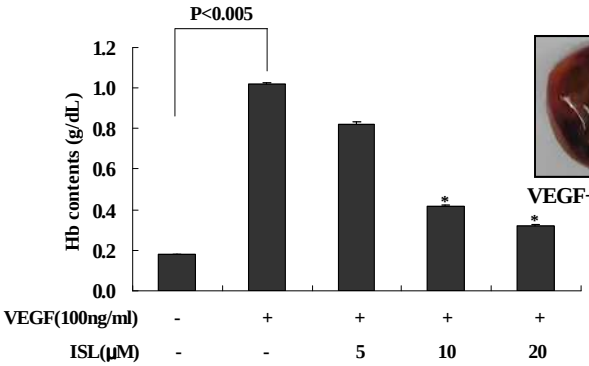
VEGF binds with different affinities to 3 related receptor tyrosine kinases: VEGFR-1 (fms-like tyrosine kinase-1), VEGFR-2 (homolog to murine fetal liver kinase-1), and VEGFR-3. VEGF mediates its activity mainly via two high-affinity receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2. We investigated the expression of two VEGF receptors in HUVECs exposed to ISL for 24 hr. VEGF-stimulated expression of VEGFR-1 and VEGFR-2 was reduced by ISL treatment (Fig. 8).

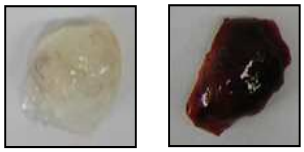
ISL reduces VEGF-induced phosphorylation of p38, ERK1/2 and JNK in HUVECs

VEGF induces the expression of various angiogenesis-related proteins in ECs. Previous data have demonstrated that ERK, JNK, and p38 members of the MAP kinase family play important roles for VEGF-induced angiogenesis[27]. Western blot analysis in this study showed that VEGF induced phosphorylation of p38, ERK1/ 2 and JNK and ISL significantly suppressed the activation of these three MAPKs (Fig. 9). Especially, ISL 20 μM completely reduced VEGF-induced phosphorylation of JNK. The protein levels of p38, ERK1/ 2 and JNK remained unchanged with ISL treatment.

A

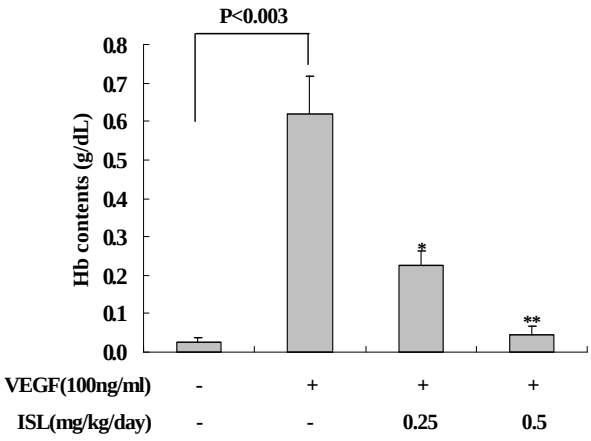
Treatment	Hb contents (g/dL)	% of inhibition
Control	0.18 ± 0.001	
VEGF 100ng/ml	1.02 ± 0.007	0
VEGF + ISL 5μM	0.82 ± 0.009	19.31
VEGF + ISL 10μM	0.42 ± 0.002	58.94
VEGF + ISL 20μM	0.32 ± 0.003	68.43

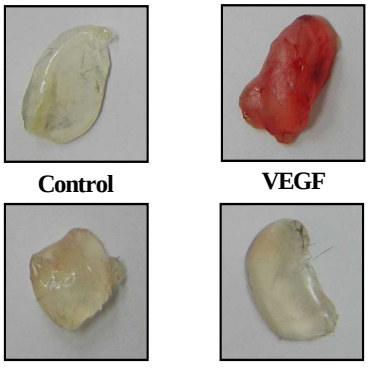




B

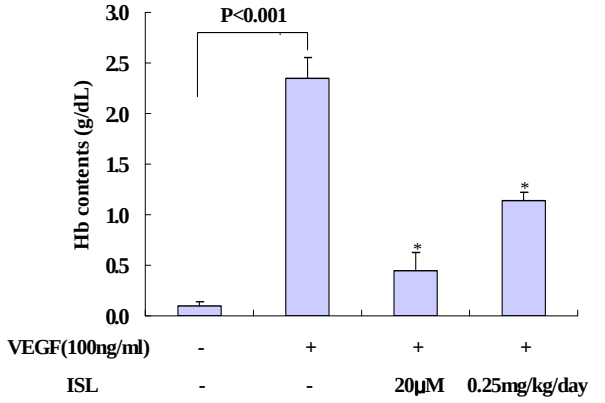
Treatment	Hb contents (g/dL)	% of inhibition
Control	0.02 ± 0.012	
VEGF 100ng/ml	0.62 ± 0.098	0
VEGF + ISL 0.25mg/kg/day	0.23 ± 0.039	63.6
VEGF + ISL 0.5mg/kg/day	0.05 ± 0.021	92.6





C

Treatment	Hb contents (g/dL)	% of inhibition
Control	0.09 ± 0.042	
VEGF 100ng/ml	2.35 ± 0.200	0
VEGF + ISL 20μM	0.44 ± 0.186	81.31
VEGF + ISL 0.25mg/kg/day	1.15 ± 0.073	51.34



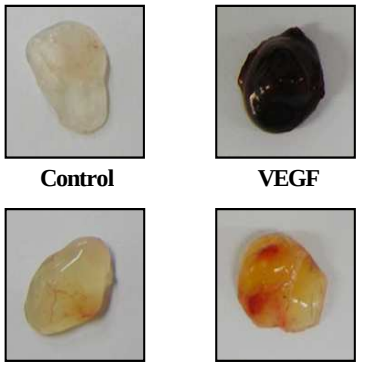


Figure 7. ISL inhibits VEGF-induced angiogenesis *in vivo* Matrigel plug assay. (A) C57/ BL6 mice were injected with 0.6 ml of Matrigel containing VEGF (100 ng/ ml) and ISL (5 - 20 μM). *, P<0.001 *versus* VEGF-treated group. (B) ISL was orally administrated for 7 days with 20-40 μM after injection of Matrigel with VEGF. *, P<0.01 ; **, P<0.005 *versus* VEGF-treated group. (C) The effect of ISL on VEGF-induced angiogenesis was compared in mice with subcutaneous injection of VEGF and ISL (20 μM) and in orally-treated mice with ISL after Matrigel injection with VEGF. *, P<0.001 *versus* VEGF-treated group. After 7 days, mice were sacrificed and Matrigel plugs were excised. Matrigel plugs were photographed. To quantify the formation of new blood vessels in Matrigel, the amount of hemoglobin in each plug was assayed with Drabkin's reagent kit according to the manufacturer's protocol.

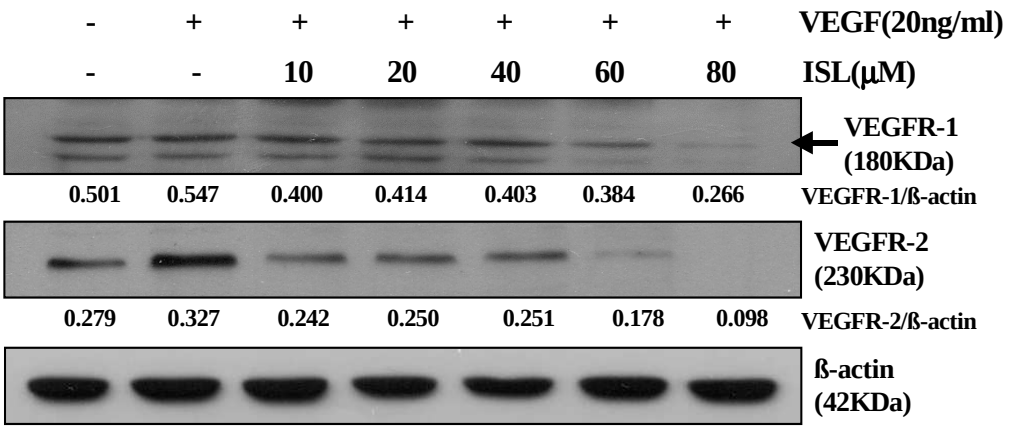


Figure 8. **ISL down-regulates VEGF-stimulated VEGFR-1 and VEGFR-2 expression in HUVECs. Serum-starved HUVECs were stimulated with VEGF (20 ng/ ml) and then incubated with vehicle (DMSO 0.1%) or ISL (10 - 80 μM) for 24 hr. The cells were scraped from the culture dishes and the resulting lysates clarified by centrifugation. Western blotting was then performed using anti-VEGFR-1 and anti-VEGFR-2 antibodies. Membranes were reprobed for β-actin. The level of VEGFR-1 and VEGFR-2 was quantified by densitometry and expressed as the ratio of VEGFR-1 or VEGFR-2/ β-actin.**

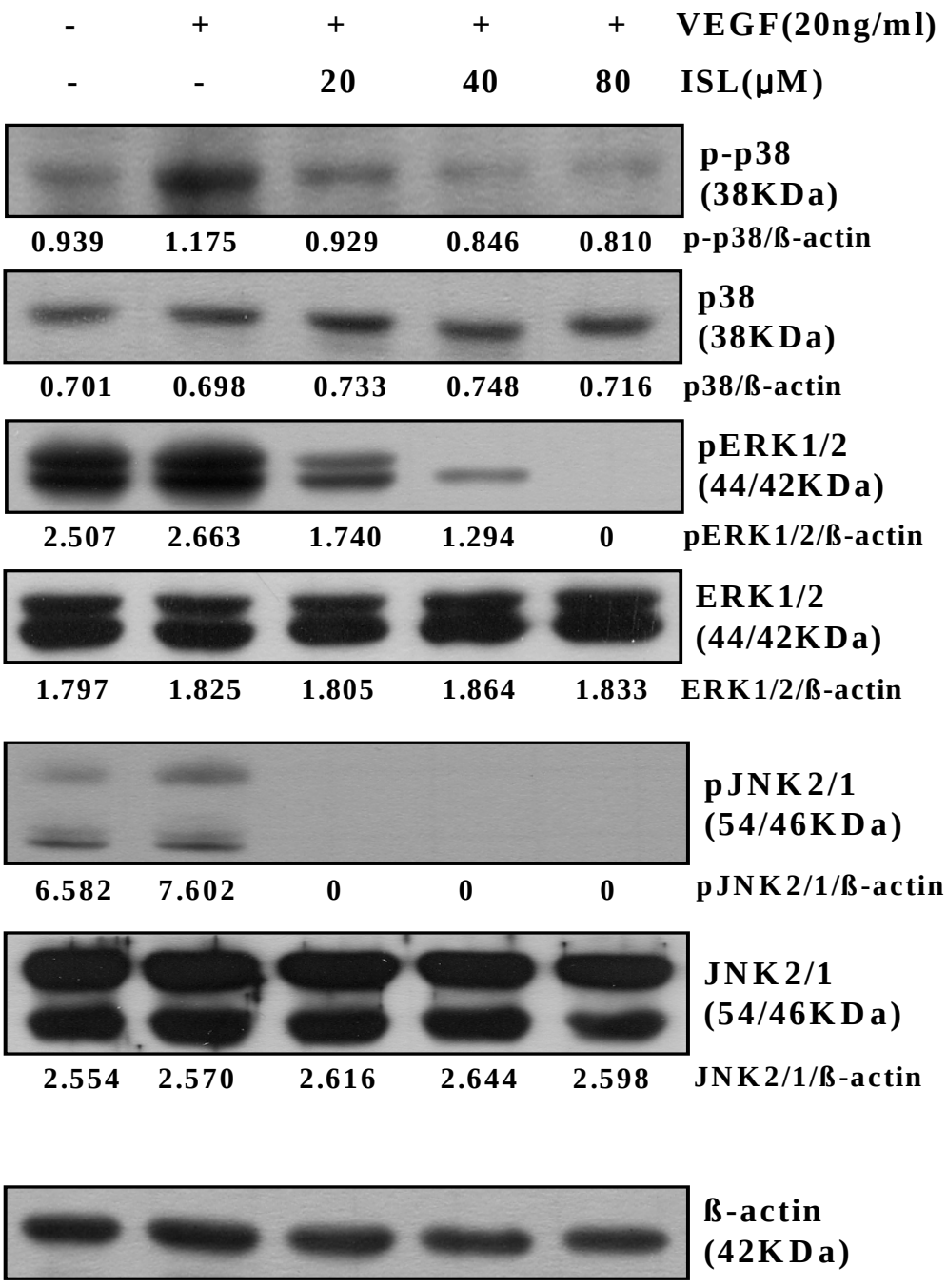

- + + + + VEGF(20ng/ml)
- - 20 40 80 ISL(μM)
p-p38 (38KDa)
0.939 1.175 0.929 0.846 0.810 p-p38/ß-actin
p38 (38KDa)
0.701 0.698 0.733 0.748 0.716 p38/ß-actin
pERK1/2 (44/42KDa)
2.507 2.663 1.740 1.294 0 pERK1/2/ß-actin
ERK1/2 (44/42KDa)
1.797 1.825 1.805 1.864 1.833 ERK1/2/ß-actin
pJNK2/1 (54/46KDa)
6.582 7.602 0 0 0 pJNK2/1/ß-actin
JNK2/1 (54/46KDa)
2.554 2.570 2.616 2.644 2.598 JNK2/1/ß-actin
ß-actin (42KDa)

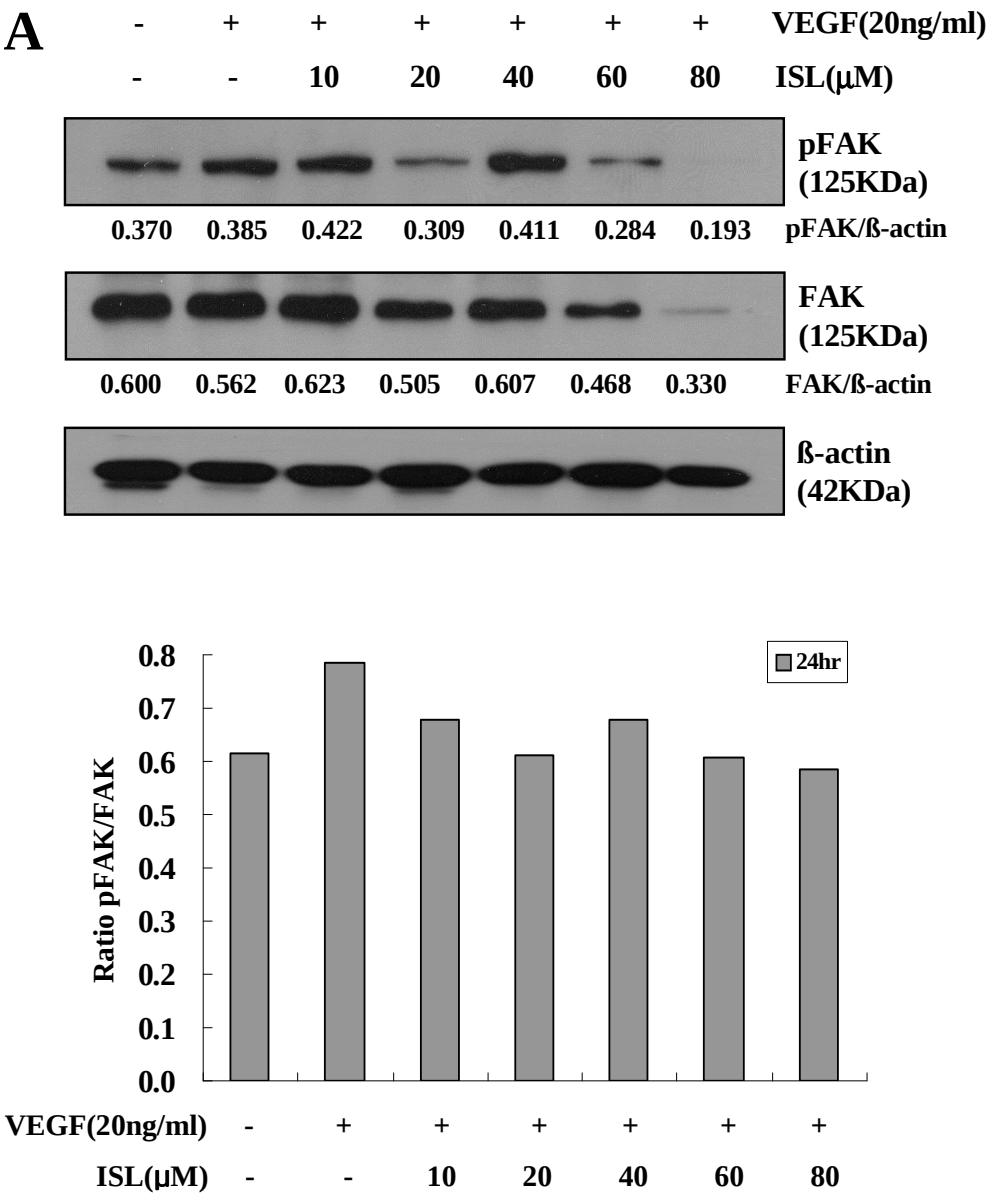
Figure 9. **ISL reduces VEGF-induced phosphorylation of p38, ERK1/ 2 and JNK in HUVECs. Serum-starved HUVECs were treated with VEGF (20 ng/ mL) in the presence or absence of ISL (20 - 80 μM) for 24 hr. Equal amounts of protein extract from cell lysates were analyzed by Western blot analysis for phospho-p38, p38, phospho-ERK1/ 2, ERK1/ 2, phospho-JNK, JNK and β-actin. The relative intensities of protein band were measured by densitometric analysis and normalized with that of β -actin.**

ISL decreases VEGF-induced activation of FAK and paxillin in HUVECs

Endothelial cell migration is an early key step in the initiation of angiogenesis[42,43]. FAK is a key transducer of signals converging from integrins and RTKs important for both survival and migration[44]. Paxillin is known to be important in connecting the integrins with actin fibers via interaction with various molecules such as vinculin and FAK[47,48]. VEGF induces tyrosine phosphorylation of FAK and the focal adhesion-associated protein paxillin to promote recruitment of FAK to new focal adhesion in HUVECs[25,46]. We analyzed the activation of FAK and paxillin when HUVECs were exposed to ISL with VEGF (20 ng/ ml) for 24 hr. VEGF stimulation significantly increased both FAK and paxillin phosphorylation. Exposure to the indicated concentration of ISL led to a dose-dependent reduction of the levels of phospho-FAK (pY397) (Fig. 10A) and phospho-paxillin (Tyr31) (Fig. 10B) as well as the expression levels of FAK and paxillin. Densitometric analysis showed that ISL inhibits phosphorylation of FAK and paxillin despite of reduction of their total protein level. These results indicate ISL decreases VEGF-induced activation of FAK and paxillin in HUVECs.

ERK1/2 and JNK are critical in VEGF-induced proliferation and DNA synthesis of HUVECs

The proliferation of endothelial cells in response to VEGF is of central importance to the angiogenesis process. To examine the roles of three MAP kinase, p38, ERK1/ 2 and JNK in VEGF-induced proliferation of HUVECs, we determined whether MAPK inhibitors suppressed cell viability and DNA synthesis in VEGF-treated HUVECs. As shown in Fig. 11A, the enhanced cell viability by VEGF (20 ng/ ml) treatment was markedly inhibited by treatment with PD98059 (a selective and cell-permeable inhibitor of the ERK1/ 2), SP600125 (a potent and selective inhibitor of JNK) and


A
- + + + + + + VEGF(20ng/ml)
- - 10 20 40 60 80 ISL(μM)
pFAK (125KDa)
0.370 0.385 0.422 0.309 0.411 0.284 0.193 pFAK/ß-actin
FAK (125KDa)
0.600 0.562 0.623 0.505 0.607 0.468 0.330 FAK/ß-actin
ß-actin (42KDa)
24hr
Ratio pFAK/FAK
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
VEGF(20ng/ml) - + + + + + +
ISL(μM) - - 10 20 40 60 80

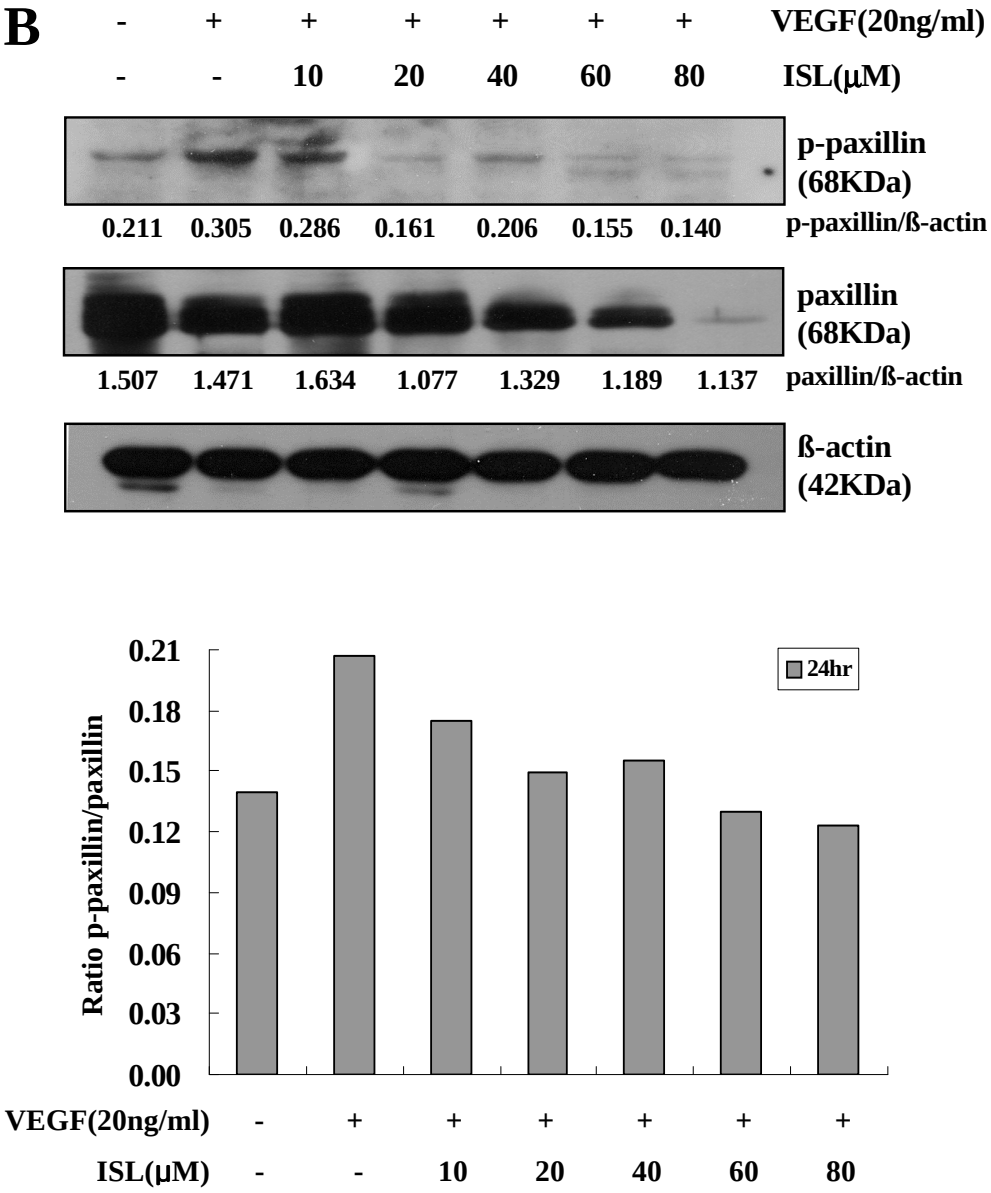

B
- + + + + + + VEGF(20ng/ml)
- - 10 20 40 60 80 ISL(μM)
p-paxillin (68KDa)
0.211 0.305 0.286 0.161 0.206 0.155 0.140 p-paxillin/ß-actin
paxillin (68KDa)
1.507 1.471 1.634 1.077 1.329 1.189 1.137 paxillin/ß-actin
ß-actin (42KDa)
Ratio p-paxillin/paxillin
0.21
0.18
0.15
0.12
0.09
0.06
0.03
0.00
24hr
VEGF(20ng/ml) - + + + + + +
ISL(μM) - - 10 20 40 60 80

Figure 10. **ISL decreases VEGF-induced activated of FAK and paxillin in HUVECs. Serum-starved HUVECs were treated with VEGF (20 ng/ mL) in the presence or absence of ISL (10 - 80 μM) for 24 hr. Equal amounts of protein extract were subjected to SDS-PAGE, transferred to PVDF, and sequentially probed with the antibodies of phospho-FAK and FAK (A), and phospho-paxillin and paxillin (B). β-actin expression was reprobed to indicate evenness of loading of protein extract from each treatment. After normalization with the intensity of β-actin, the ratio of phosphorylated protein to total protein was determined by comparing the relative intensities of protein band.**

SB203580 (a highly specific and cell-permeable inhibitor of p38 MAP kinase). Moreover, PD98059 and SP600125, not SB203580, blocked VEGF-stimulated DNA synthesis in HUVECs (Fig. 11B). Our results demonstrate that ERK1/2 and JNK, not p38 MAP kinase, play an important role for VEGF-induced proliferation of HUVECs.

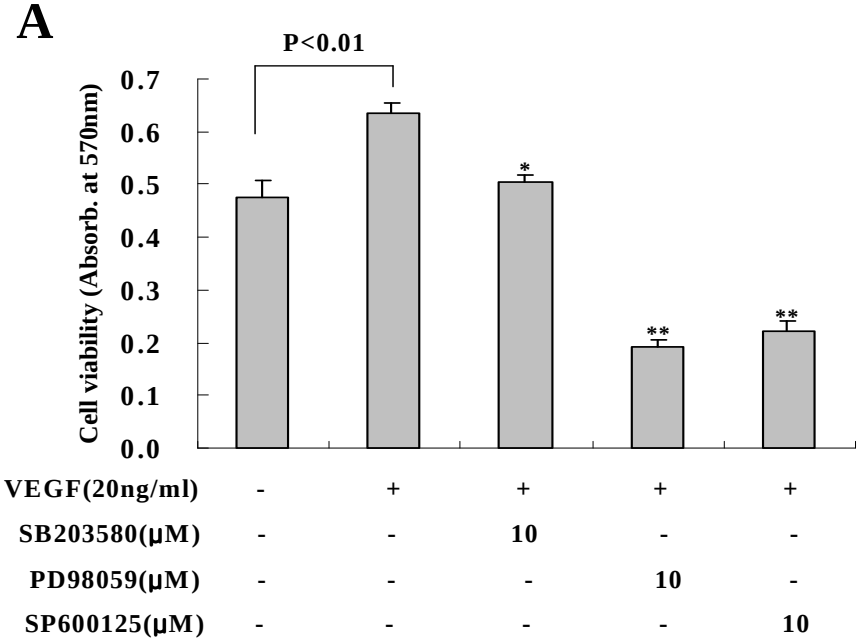
VEGFR-1 and/or VEGFR-2 regulate VEGF-induced phosphorylation of p38, ERK1/2 and JNK in HUVECs

In HUVECs, VEGF-induced activation of ERK and p38 MAPK, closely associated with cell proliferation and migration, is mostly mediated by VEGFR-2[13,50,51]. To determine VEGF receptors inducing the activation of respective MAPKs, the cells were exposed to indicated concentrations of anti-VEGFR-1 mAb or anti-VEGFR-2 mAb 2 hr before the addition of VEGF (20 ng/ml). 24 hr later, total protein was extracted. Western blot analysis showed that VEGF-induced phosphorylation of p38, ERK1/2 and JNK was significantly inhibited by anti-VEGFR-2 mAb (Fig. 12). Anti-VEGFR-1 mAb did not affect the phosphorylation of ERK1/2, but partially inhibited VEGF-induced phosphorylation of p38 and almost completely abrogated VEGF-induced phosphorylation of JNK, at 10 μg/ml. These results VEGFR-2 was a key receptor for phosphorylation of p38, ERK1/2 and JNK in HUVECs stimulated with VEGF.

VEGF-induced FAK and paxillin phosphorylation are dependent on ERK1/2 and JNK pathway via VEGFR-2 in HUVECs

VEGF induces tyrosine phosphorylation of FAK and paxillin[24] and requires FAK to promote new focal adhesion formation in HUVECs[25]. We examined whether VEGF-induced phosphorylation of FAK and paxillin depend on VEGFR-1 or

A


P<0.01
Cell viability (Absorb. at 570nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
**
**
VEGF(20ng/ml) - + + + +
SB203580(μM) - - 10 - -
PD98059(μM) - - - 10 -
SP600125(μM) - - - - 10

B

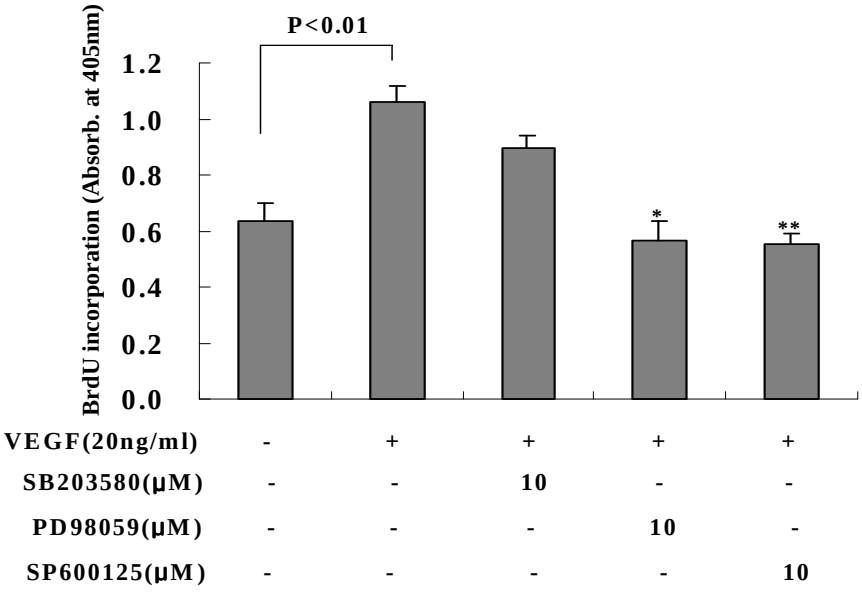

P<0.01
BrdU incorporation (Absorb. at 405nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
VEGF(20ng/ml) - + + + +
SB203580(μM) - - 10 - -
PD98059(μM) - - - 10 -
SP600125(μM) - - - - 10

Figure 11. ERK1/ 2 and JNK are critical in VEGF-induced proliferation of HUVECs. Cells were treated with SB203580 (10 μM), PD98059 (10 μM), and SP600125 (10 μM) in the presence of VEGF (20 ng/ ml) for 24 hr. A, The viability was measured using MTT assay. *, P<0.005 ; **, P<0.001 *versus* VEGF-treated group. B, DNA synthesis was assessed by BrdU incorporation assay. *, P<0.01 ; **, P<0.001 *versus* VEGF-treated group.

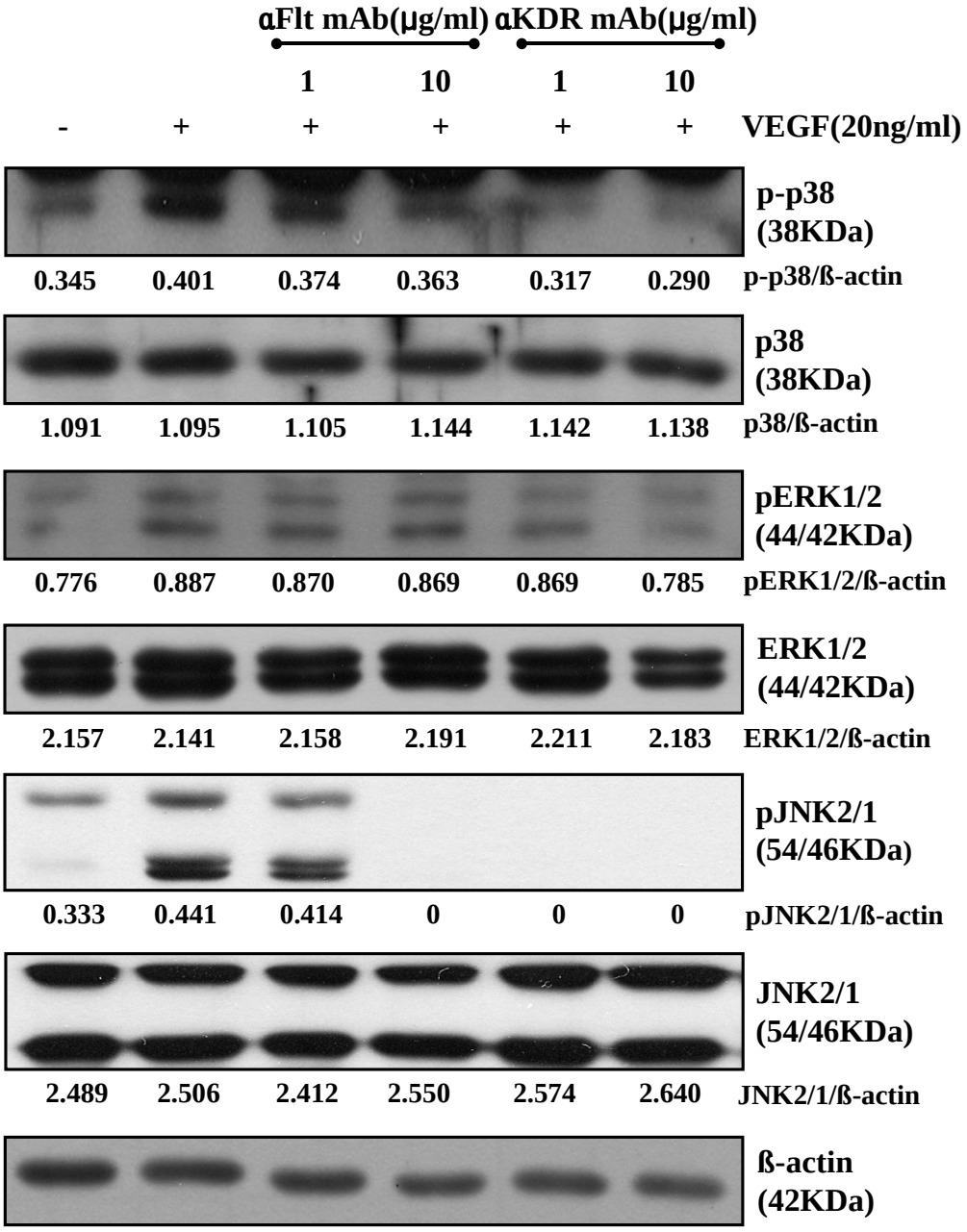

αFlt mAb(μg/ml)
αKDR mAb(μg/ml)
1
10
1
10
-
+
+
+
+
+
VEGF(20ng/ml)
p-p38
(38KDa)
0.345
0.401
0.374
0.363
0.317
0.290
p-p38/ß-actin
p38
(38KDa)
1.091
1.095
1.105
1.144
1.142
1.138
p38/ß-actin
pERK1/2
(44/42KDa)
0.776
0.887
0.870
0.869
0.869
0.785
pERK1/2/ß-actin
ERK1/2
(44/42KDa)
2.157
2.141
2.158
2.191
2.211
2.183
ERK1/2/ß-actin
pJNK2/1
(54/46KDa)
0.333
0.441
0.414
0
0
0
pJNK2/1/ß-actin
JNK2/1
(54/46KDa)
2.489
2.506
2.412
2.550
2.574
2.640
JNK2/1/ß-actin
ß-actin
(42KDa)

Figure 12. **VEGFR-1 and/ or VEGFR-2 regulate VEGF-induced phosphorylation of p38, ERK1/ 2 and JNK in HUVECs. HUVECs were exposed to indicated concentrations of αFlt mAb (monoclonal antibody against VEGFR-1) or αKDR mAb (monoclonal antibody against VEGFR-2) 2 hr before the addition of VEGF. After the cultures were incubated with or without VEGF (20 ng/ ml) for 24 h, equal amounts of protein extract were subjected to SDS-PAGE, transferred to PVDF, and sequentially probed with antibodies of phospho-p38 and p38, phospho-ERK1/ 2 and ERK1/ 2, and phospho-JNK and JNK. β-actin expression was reprobed to indicate evenness of loading of protein extract from each treatment. The intensity of protein was measured by densitometric analysis and normalized with that of β-actin.**

VEGFR-2. VEGF-induced phosphorylation of FAK and paxillin was completely inhibited by anti-VEGFR-2 mAb (Fig. 13), indicating that VEGFR-2 was responsible for phosphorylation of FAK and paxillin in HUVECs stimulated with VEGF.

Because the phosphorylation of p38, ERK1/2 and JNK as well as that of FAK and paxillin was inhibited by blocking VEGFR-2, we next examined the role of these three MAPKs in mediating activation of FAK and paxillin by VEGF. When HUVECs in serum-free medium were treated with 10 μM of SB203580, PD98059 or SP600125 and 20 ng/ml VEGF for 24 hr, VEGF-induced phosphorylation of FAK and paxillin was completely decreased by PD98059, ERK1/2 inhibitor, and SP600125, JNK inhibitor, in HUVECs (Fig. 14). In contrast, SB203580 did not affect phosphorylation of FAK and paxillin induced by VEGF.

Moreover, we examined the effect of ZM323881, a cell-permeable and potent inhibitor of VEGFR-2 tyrosine kinase activity, on FAK and paxillin activation. FAK and paxillin phosphorylation induced by VEGF were strongly inhibited by ZM323881 treatment for 24 hr (Fig. 14). These results indicate that VEGF-induced FAK and paxillin phosphorylation in HUVECs induces by (or is due to) VEGFR-2-mediated activation of ERK1/2 and JNK.

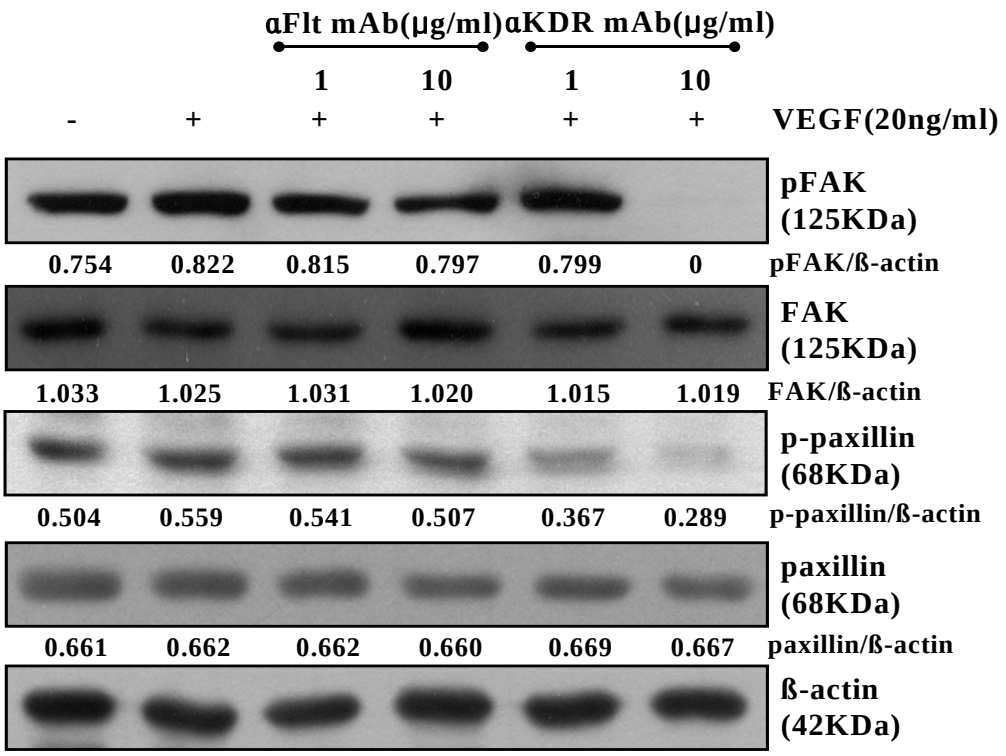


Figure 13. VEGFR-2 is responsible for VEGF-induced phosphorylation of FAK and paxillin in HUVECs. HUVECs were exposed to indicated concentrations of αFlt mAb (monoclonal antibody against VEGFR-1) or αKDR mAb (monoclonal antibody against VEGFR-2) 2 hr prior to the addition of VEGF. Then the cultures were incubated with or without VEGF (20 ng/ ml) for 24 hr and the expression phospho-FAK and FAK, and phospho-paxillin and paxillin were analysed by Western blot. β-actin expression was reprobed to indicate evenness of loading of protein extract from each treatment. The intensity of protein was measured by densitometric analysis and normalized with that of β-actin.

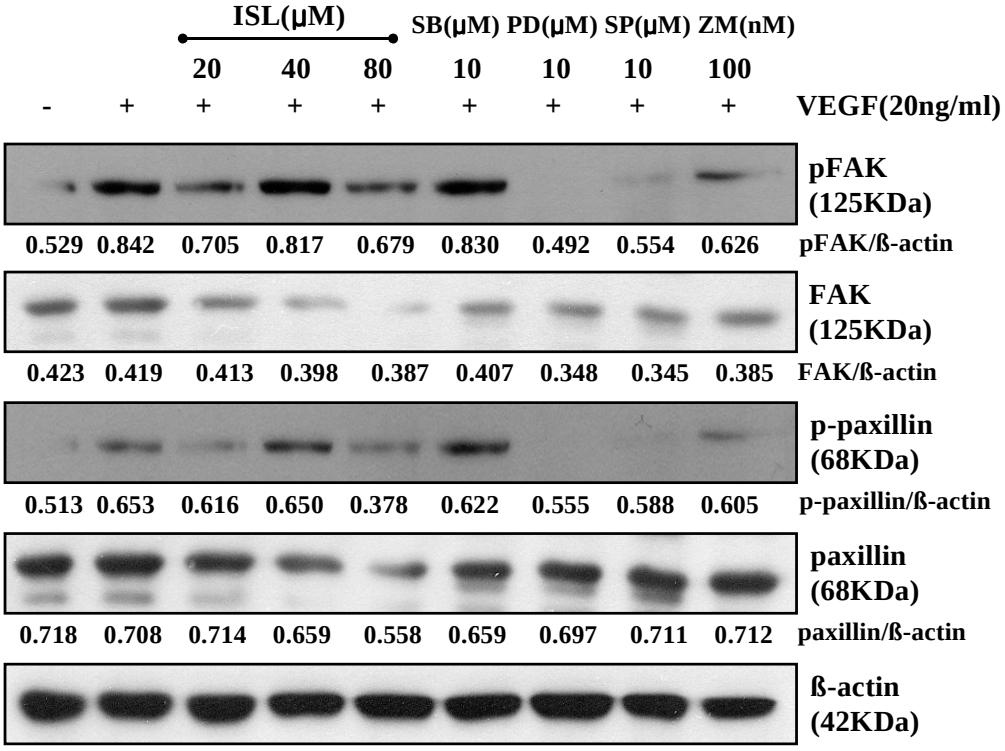


Figure 14. **VEGF-induced FAK and paxillin phosphorylation are dependent on VEGFR-2, ERK1/ 2 and JNK pathway in HUVECs.** Serum-starved HUVECs were incubated with vehicle (DMSO 0.1%), ISL (20 - 80 μM), SB203580 (10 μM), PD98059 (10 μM), SP600125 (10 μM), and ZM323881 (100nM) and stimulated with VEGF (20 ng/ ml) for 24 hr. The cells were scraped from the culture dishes and the resulting lysates clarified by centrifugation. Western blotting using anti-pFAK, anti-FAK, anti-p-paxillin and anti-paxillin antibodies was performed. Membranes were reprobed for β-actin.

Ⅳ. DISCUSSION

In an attempt to identify phytochemicals contributing to the well documented preventive effect of plant-based diets on cancer incidence and mortality[55,56], it has been shown previously that certain isoflavonoids and flavonoids inhibit proliferation of cultured tumor cells and in vitro angiogenesis[57]. It has been also reported that endothelial cell proliferation and migration in response to VEGF play an important role in angiogenesis, which is essential for the tumors to enlarge and metastasize. Recently, a significant reduction in the VEGF-induced angiogenesis, neovascular survival, and growth of human tumor xenografts, was obseved after once-daily oral administration of AZD2171, a highly potent inhibitor of VEGFR-2 tyrosine kinase, at comparatively low doses[58]. In the present study, we showed that isoflavonoid ISL inhibited VEGF-induced proliferation, DNA synthesis, migration and tube formation in HUVECs. In agreement with the inhibiton of angiogenesis in vitro, ISL inhibited VEGF-induced angiogenesis in a dose-dependent manner in vivo Matrigel plug assay. Moreover, oral daily administration of ISL inhibited angiogenesis induced by VEGF in mice. These results suggest that ISL is capable of inhibiting VEGF-dependent angiogenesis and that it is also effective as an orally bioavailable agent.

The modulation of VEGF-induced angiogenesis has great therapeutic potential to induce wound healing or impede tumor-related growth[59] and proliferative diabetic retinopathy[60]. VEGF is an important, multifunctional angiogenic cytokine that exerts a variety of biological activities on vascular endothelium. These include induction of microvascular hyper-permeability, stimulation of proliferation and migration, significant reprogramming of gene expression, endothelial cell survival, and prevention of senescence[22,61]. All of these functions are thought to be mediated by two receptor tyrosine kinases, VEGFR-1 and VEGFR-2, that are selectively expressed on vascular endothelium and up-regulated at sites of VEGF overexpression as in tumors,

healing wounds, chronic inflammation, etc.[42]. Because both receptors are expressed on vascular endothelium, it has been difficult to define the respective role in mediating the various signaling events and biological activities induced by VEGF in endothelium. Therefore, we first observed that ISL reduced VEGF-stimulated expression of VEGFR-1 and VEGFR-2 in HUVECs. Furthermore, the finding that ISL significantly inhibited VEGF-induced phosphorylation of p38, ERK1/ 2 and JNK in a dose-dependent manner. Our results provide the anti-angiogenic potential of ISL suppressing VEGF-induced angiogenesis by downregulating of the expression of two VEGFRs and blocking MAPKs activation in VEGF-stimulated HUVECs. So, two possibilities present themselves : (a) ISL binds to multiple sites on the cell surface and, consequently, results in darken VEGF receptors expression through blocks VEGF from binding to VEGFRs ; or (b) ISL binds directly to the VEGF binding site of VEGFR, blocking the binding of VEGF.

To explain the molecular mechanism underlying antiangiogenic activity of ISL, we first determined the intracellular signal of VEGFR-1 and VEGFR-2 coupled to MAPKs. The current information has been gleaned largely from studies with several cell lines that VEGFR-2 activation is sufficient to stimulate SAPK2/ p38[53]. In HUVECs, a VEGFR-2 neutralizing antibody inhibited the activation of SAPK2/ p38. Moreover, PlGF, a specific ligand for VEGFR-1 that does not activate VEGFR-2[21], did not stimulate the SAPK2/ p38 pathway. In PAE cells that express only VEGFR-2 or VEGFR-1, SAPK2/ p38 was activated in the PAE/ VEGFR-2 transfectants but not in the PAE/ VEGFR-1 cells. In contrast, a recent study demonstrated that VEGFR-1 activates p38 MAP kinase, regulating actin reorganization[29]. It has been more recently appreciated that various growth factor receptors including VEGFRs stimulate both ERK and JNK[37,17-19], leading to the proposal that both may contribute to cell proliferation. VEGFR-2, not VEGFR-1, was involved in ERK activation by VEGF[20]. Activation of JNK1/ 2 is required for a VEGFR-3-dependent prosurvival signaling[16]. However, the roles of VEGFR-1 and VEGFR-2 for JNK activation in VEGF-stimulated

HUVECs remain to be elucidated. In our study, VEGF-induced phosphorylation of p38 was partially or completely abolished by the anti-VEGFR-1 mAb and anti-VEGFR-2 mAb, respectively, indicating that VEGF-induced phosphorylation of p38 in HUVECs is critically dependent on VEGFR-2 and is slightly reliant on VEGFR-1. VEGF-induced phosphorylation of ERK1/ 2 and JNK was abolished by the anti-VEGFR-2 mAb, whereas VEGF-induced phosphorylation of JNK was completely inhibited by the anti-VEGFR-1 mAb (10 μg/ ml) and anti-VEGFR-2 mAb, suggesting that VEGF-induced phosphorylation of ERK1/ 2 in HUVECs is critically dependent on VEGFR-2, whereas VEGF-induced phosphorylation of JNK is dependent on both VEGFR-1 and VEGFR-2. Our data demonstrates that the VEGFR-2 is a major receptor mediating VEGF-induced phosphorylation of p38, ERK1/ 2 and JNK in response to VEGF, although VEGF-induced phosphorylation of p38 and JNK is delicately reliant on VEGFR-1. Our results showed that treatment of HUVECs with PD98059 or SP600125 inhibited VEGF-induced proliferation and DNA synthesis, while SB203580 didn't significantly affect not only HUVEC proliferation but also DNA synthesis. These findings suggest that VEGF-induced HUVEC proliferation and DNA synthesis takes place through VEGFR-2-mediated ERK1/ 2 and JNK activation and VEGFR-1 may induce cell proliferation by activating JNK in response to VEGF. ISL inhibits VEGF-induced angiogenesis by reducing VEGFR-1 and VEGFR-2 expression and ERK1/ 2 and JNK activation, suppressing the proliferation of HUVECs.

In recent years, numerous studies have reported on the tyrosine phosphorylation of cellular components as a key transducer of integrin-generated signaling pathways[54]. Consistent with a recent report using NIH3T3 cells[49], cell adhesion and growth factor stimulation triggered distinct initial tyrosine phosphorylation events in endothelial cells. Tyrosine phosphorylation of FAK and paxillin requires anchorage to a matrix substratum. It has been reported that when endothelial cells migrate into a wounded area on tissue culture plastic, FAK tyrosine phosphorylation is induced[22], suggesting an important role for FAK in endothelial cell migration. This finding is consistent

with report that stimulation of the FAK/ paxillin pathway in HUVECs was accompanied by a marked VEGF-induced increase in the localization of both FAK and paxillin to focal adhesions and filamentous structures[25]. Here, we found that ISL was markedly inhibited phosphorylation of FAK and paxillin in HUVECs by ISL despite reduction in their protein levels. FAK is a substrate for the calcium-activated neutral protease calpain[41]. FAK also degrades during apoptosis by activated caspase[39]. In our study, although the mechanism of FAK and paxillin degradation is not determined, an activated caspases or protease calpain may cause degradation of FAK and paxillin in HUVECs.

VEGF elicited actin reorganization and vinculin assembly in focal adhesion plaque of HUVECs. Anti-VEGFR-1 mAb completely abrogated actin reorganization, but failed to affect vinculin assembly[45]. On the contrary, anti-VEGFR-2 mAb inhibited vinculin assembly, but failed to affect actin reorganization[45]. VEGF induced phosphorylation of FAK and paxillin[29]. Anti-VEGFR-1 mAb exhibited no effect on phosphorylation of FAK or paxillin, whereas anti-VEGFR-2 mAb completely inhibited phosphorylation of FAK and paxillin[29]. Therefore, previous studies indicate that VEGFR-1-mediated signal regulates actin reorganization, whereas VEGFR-2-mediated signal regulates vinculin assembly and VEGF-induced phosphorylation of FAK and paxillin, both of which are required for cell migration. Consistent with these findings, our results showed that VEGF-induced phosphorylation of FAK and paxillin on endothelial cell is critically dependent on VEGFR-2. Furthermore, pretreatment of HUVECs with SB203580 inhibited VEGF-induced proliferation[52].

Recent studies in HUVECs indicate that VEGFR-2 was upstream of FAK activation was mainly supported by the observation that the VEGFR-2 neutralizing antibody inhibited tyrosine phosphorylation of FAK. Activation of FAK by VEGFR-2 was independent of p38 and ERK1/ 2 activation[10]. Our present study clearly indicates that VEGF-induced phosphorylation of FAK and paxillin was regulated preferentially via an ERK1/ 2 and JNK-dependent and p38-independent pathway. Especially, the

present study is first report demonstrating that JNK plays an important role in VEGF-induced cell proliferation and migration of HUVECs.

Ⅴ. CONCLUSION

Previous studies have demonstrated that oral administration of ISL suppresses the induction of preneoplastic aberrant crypt foci (ACF) in the male F344 rat colon when given in the initiation and/ or post-initiation stages, with the most pronounced effects being observed in the case of administration in both stages[36]. Moreover, animal and cell culture model studies indicated that ISL inhibited tumour formation, carcinogenesis and metastasis[26,38]. Considering that angiogenesis is essential for tumor growth, the anti-tumor effect of ISL may be correlated with its anti-angiogenic activity. Using in vivo angiogenesis model, the anti-angiogenic activities of ISL were evaluated. ISL or oral administration of ISL remarkably suppressed induction of new blood formation in Matrigel plug implanted in C57BL/ 6 mice in response to VEGF. These anti-angiogenic activities of ISL may be explained by its inhibitory action on proliferation, migration, and tube formation of HUVECs in response to VEGF.

In conclusion, we demonstrate that ISL acts as a potent angiogenesis inhibitor. As illustrated in Fig. 15, ISL inhibits VEGF-mediated angiogenesis by regulating the distinct signalling of VEGFR-1 and VEGFR-2 via MAPKs and FAK/ paxillin in HUVECs.

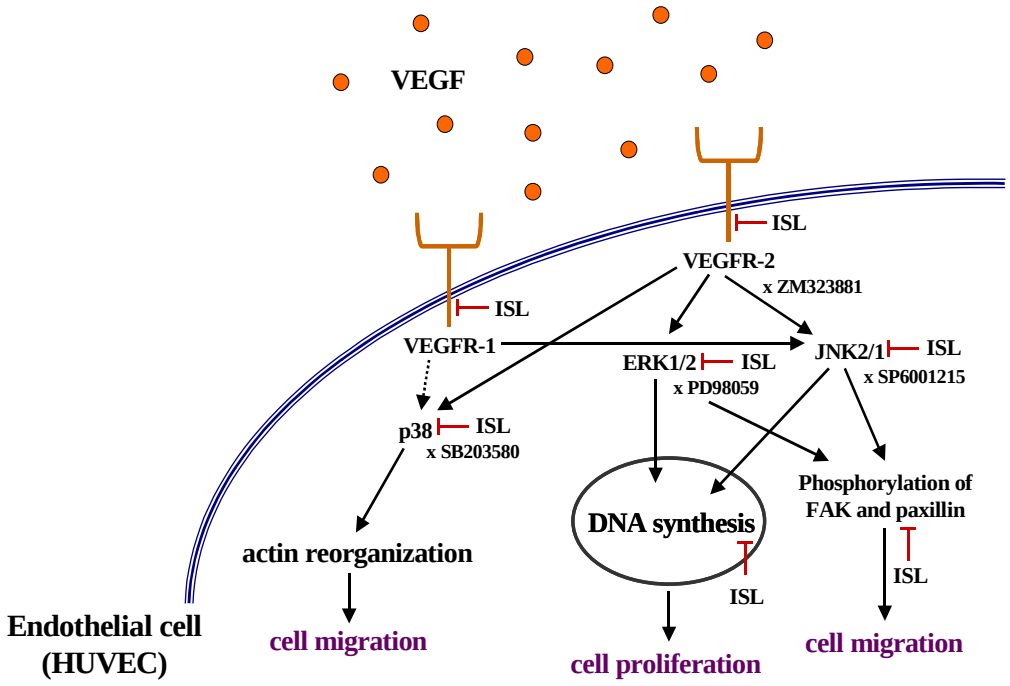


Figure 15. ISL inhibits VEGF-mediated angiogenesis by down-regulating the distinct signalling of VEGFR-1 and VEGFR-2 via MAPKs and FAK/ paxillin in HUVECs. VEGFR-2 mediates the signals for DNA synthesis and phosphorylation of FAK/ paxillin via ERK1/ 2-dependent and JNK2/ 1-dependent pathway. VEGFR-1 mediates p38 activation to regulate cell migration via actin reorganization and JNK activation.

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국 문 초 록

이소리퀴리티제닌은 인간 혈관내피세포에서 **MAPKs**를 통해 혈관내피성장인자 수용체들의 신호전달체계를 조절함으로써 혈관내피성장인자로 유도한 신생혈관생성을 억제한다

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손 승 화

종양이 성장하기 위해서는, 종양으로부터 생성된 신진대사 노폐물의 제거와 적절한 영양공급원이 필요한데 이를 위해서 종양세포는 기존의 혈관으로부터 새로운 혈관을 형성하는 신생혈관생성을 유도한다. 신생혈관생성은 **pro-angiogenic factor**와 **anti-angiogenic factor**에 의해 조절되는데, 종양 성장에 있어서 강력한 조절 포인트로써 인식되어지고 있다. 많은 **pro-angiogenic factor** 중에 가장 유력한 인자는 혈관내피성장인자/ 혈관투과성인자 **(vascular endothelial growth factor, VEGF)** 이다. 혈관내피성장인자는 여러 종양세포에 의해 분비되며 분비된 혈관내피성장인자에 의해 기질 분해 및 내피세포의 증식과 이동이 촉진되어 새로운 혈관이 형성된다. 따라서 혈관내피성장인자는 신생혈관생성억제를 위한 좋은 표적으로 많은 연구가 진행되었다. 혈관내피성장인자는 일차 배양한 혈관내피에서 선택적으로 발현되는 혈관내피성장인자 수용체 **1**과 **2**에 의해 다양한 기능을 수행한다. 그러므로, 혈관내피성장인자와 그 수용체간의 결합을 막는 것이 **anti-angiogenesis** 치료를 통해 암세포의 성장과 신

생혈관생성을 억제하는데 매우 중요하다.

본 연구에서 이소리퀴리티제닌이 인간 혈관내피세포에서 혈관내피성장인자로 유도한 세포의 증식, **DAN** 합성, 세포의 이동 그리고 튜브 형성을 농도 의존적으로 억제하였다. 생체내에서 이소리퀴리티제닌의 신생혈관생성 억제활성은 **Matrigel plug assay**를 수행하여 확인하였다. 이소리퀴리티제닌이 신생혈관생성 억제효능을 나타내는 작용기작을 규명하기 위해, 혈관내피성장인자로 촉진된 인간 혈관내피세포의 증식과 이동을 조절하는 혈관내피성장인자 수용체 **1** 및 수용체 **2** 매개 신호전달체계에 대한 영향을 조사하였다. 이소리퀴리티제닌은 혈관내피성장인자를 처리한 인간 혈관내피세포에서 혈관내피성장인자 수용체 **1**, 수용체 **2, FAK** 그리고 **paxillin**의 발현뿐만 아니라 **MAPKs, FAK** 그리고 **paxillin** 의 인산화도 억제하였다. 먼저 혈관내피성장인자가 수용체를 통해 세포의 증식과 이동을 촉진하는 세포신호전달체계를 조사한 결과, 혈관내피성장인자 수용체 **2**가 매개된 **ERK1/ 2**와 **JNK**의 활성화는 혈관내피성장인자가 처리된 인간제대정맥내피세포에서 세포의 증식과, **FAK**과 **paxillin**의 인산화를 통한 세포의 이동을 증가시켰다. 또한 혈관내피성장인자 수용체 **2**는 **actin reorganization**과 세포 이동의 원인이 되는 **p38 MAP kinase**의 활성화를 촉진시켰다. 뿐만 아니라, 혈관내피성장인자는 혈관내피성장인자 수용체 **1**을 통해서도 **JNK**와 **p38 MAP kinase** 활성화 증가시켜 세포 증식과 이동을 유도하는 것으로 보였다. 종합해보면, 이소리퀴리티제닌은 혈관내피성장인자로 유도된 혈관내피세포에서 혈관내피성장인자 수용체 **2** 매개 **MAPKs** 활성을 억제시킴으로써 세포의 증식과 이동을 억제하며, 또한 혈관내피성장인자 수용체 **1**을 통한 **p38 MAPKs** 활성을 억제함으로써 세포의 이동을 억제하는 것으로 생각된다.

핵심어 : 이소리퀴리티제닌, 신생혈관형성, 혈관내피성장인자, 혈관내피성장인자 수용체, 인간 혈관내피세포