The Antiangiogenic Activity of Xanthorrhizol and Curcumin and Underlying Mechanisms

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The Antiangiogenic Activity of Xanthorrhizol and Curcumin and Underlying Mechanisms

Directed by Professor Kwang-Kyun Park

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다시 한번 모든 분들께 감사드립니다..

2006년 12월 15일

북적대는 실험실 한켠에서...

김미정..

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ABSTRACT

The Antiangiogenic Activity of Xanthorrhizol and Curcumin and Underlying Mechanisms

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(Directed by Professor Kwang-Kyun Park)

Angiogenesis plays a crucial role in tumor growth and metastasis. Vascular endothelial cell proliferation and migration are critical steps in angiogenesis and are regulated by various growth factors such as vascular endothelial growth factor (VEGF). The serine/threonine kinase, Akt, is a downstream effector of phosphatidylinositol 3-kinase (PI3k), which is recognized as the major mediator of survival signals. Akt-dependent phosphorylation of the endothelial nitric oxide synthase (eNOS) at Ser 1177 plays a role in angiogenesis signaling. Increased expression of cell adhesion molecules has also been implicated in endothelial cell migration. Xanthorrhizol, a sesquiterpenoid isolated from the rhizome of *Curcuma xanthorrhiza* Roxb, has been traditionally used in Indonesia for culinary and medicinal purpose. Curcumin, which is a phenolic natural product isolated from the rhizome of *Curcuma longa* L, gives a specific flavor and color to curry. It has also been used traditionally in folk medicine to treat inflammatory disorders. It has also been used traditionally in folk medicine to treat inflammatory disorders. In this study, we

showed the antiangiogenic activity of xanthorrhizol and curcumin using *in vitro* and *in vivo* assay systems. Xanthorrhizol and curcumin inhibited VEGF-induced proliferation, chemotactic motility, and the formation of capillary-like tubes in primary cultured human umbilical vein endothelial cells (HUVECs). Both compounds also inhibited the VEGF-induced phosphorylation of Akt and eNOS via the PI3k signaling pathway in HUVECs. The VEGF-induced expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin in HUVECs was inhibited by xanthorrhizol and curcumin mainly via blockade of the NF-κB pathway. Moreover, xanthorrhizol and curcumin exerted antiangiogenic activities in mouse Matrigel plug assay and the Chorioallantoic membrane (CAM) assay.

Key words : xanthorrhizol, curcumin, human umbilical vein endothelial cells (HUVECs), angiogenesis, PI3k/Akt/eNOS, adhesion molecules, NF-κB

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I. INTRODUCTION

Angiogenesis, the growth of new blood vessels from pre-existing capillaries, is essential for tumor progression and metastasis.[1,2] In physiological conditions, the proliferation of endothelial cells is tightly regulated and self-limited.[3] In certain pathological conditions, however, angiogenesis significantly increases and loses the capacity to regulate itself. The most important manifestation of pathological angiogenesis can be observed during the development of solid tumors.[2] The modulation of tumor angiogenesis using different agents has become a highly active area of investigation in cancer research.[4]

Although a complex molecular interplay has been reported in the underlying mechanisms of angiogenesis, several lines of evidence have recently suggested the possible involvement of the phosphatidylinositol 3-kinase (PI3k)-Akt signaling

pathway in angiogenesis.[5] The PI3k-Akt signaling pathway has been shown to be activated by various growth factors, such as insulin, platelet-derived growth factor and interleukins, as well as by vascular endothelial growth factor (VEGF), a proangiogenic factor.[6] It was previously reported that the constitutive activity of PI3k induced angiogenesis and that the inhibition of PI3k signaling interfered with angiogenesis.[7]

The serine-threonine kinase, Akt, is a downstream target of PI3k. Akt is regulated by the lipid products of PI3k binding to its pleckstrin homology domain and by phosphorylation of the Thr-308 and Ser-473 residues by two phosphoinositide-dependent protein kinases, PDK1 and PDK2.[8] Akt controls cell survival, glycogen metabolism, cellular transformation, and myogenic differentiation.[9,10,11] Activated Akt has been shown to phosphorylate the proapoptotic Bcl-2 family member, Bad, caspase-9, Forkhead family transcription factor, FKHRL1, and IκB kinase, preventing apoptosis and possibly leading to endothelial cell survival.[12] Endothelial nitric oxide synthase (eNOS) was also reported to be an Akt substrate and is activated by Akt-dependent phosphorylation at Ser 1177, which results in the release of nitric oxide (NO) in endothelial cells.[13] NO has diverse biological functions and has been shown to regulate endothelial cell growth[14], apoptosis[15], migration[16,17], etc. and is essential for angiogenesis.[18,19] Recently, a growing body of evidence supports the involvement of NO in VEGF-induced angiogenesis. NOS inhibitors block VEGF-induced endothelial cell migration, proliferation, and tube formation *in vitro* as well as VEGF-induced angiogenesis *in vivo*.[20,21] VEGF is known to induce Akt-dependent phosphorylation production of eNOS,[22] resulting in the activation of eNOS and the subsequent increase in NO.

VEGF is an endothelial cell-specific mitogen and chemotactic agent that is involved in wound repair, the angiogenesis of ischemia tissue, tumor growth, microvascular permeability, vascular protection, and hemostasis.[23,24] VEGF has been shown to activate a number of different intracellular signaling pathways, including

protein kinase C, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)[(MEK)1/2], p38 MAPK, phospholipase Cγ, and phosphatidylinositol 3-kinase (PI3k)/Akt.[25] In addition to its mitogenic and chemotactic effects, VEGF induces endothelial cell survival, proliferation, migration, tube formation, and NO production.[26]

Curcuma xanthorrhiza, called Temulawak in Indonesia, has been traditionally used for the treatment of the liver disease, constipation, diarrhea, hemorrhoids, and skin eruptions in South Asian countries. Its anti-inflammatory, anti-tumor, and hepatoprotective activities have recently been reported.[27] Xanthorrhizol (Figure 1) is a sesquiterpenoid, isolated from the rhizome of *Curcuma xanthorrhiza* Roxb, which has also been used for food and medicinal purposes. Xanthorrhizol has shown potent antibacterial activity against *Streptococcus* species and oral pathogens, as reported by Hwang and others.[28,29] Curcumin (Figure 1), a major constituent of the turmeric powder extracted from the rhizomes of *Curcuma longa* L, is used as a spice to give the specific flavor and yellow color to curry and has been shown to possess potent anti-inflammatory and chemopreventive properties. Curcumin possesses antiangiogenic activities against tumor cells *in vitro*.[30,31] and inhibits tumor promotion in skin, oral, intestinal and colon carcinogenesis.[32,33,34] Information on the effects of curcumin on the regulation of angiogenesis, however, is very limited. Curcumin also suppresses several key elements in cellular signal transduction pathways including NF-κB, c-Jun/AP-1 activation, and phosphorylation reactions catalyzed by protein kinases.[35,36,37]

In certain diseases, including inflammation, atherosclerosis, pathologic angiogenesis, and vascular injury, various adhesive interactions between endothelial cells and the constituents of the blood or extracellular matrix are changed. Adhesion molecules are heavily involved in these disease processes.[38] To date, four families of cell adhesion molecules (CAMs) have been described: integrins, immunoglobulin superfamily members, cadherins, and selectins. Members of each family have been

detected in blood vessels during angiogenesis and inflammation.[39] The intercellular adhesion molecule-1 (ICAM-1) and the vascular cell adhesion molecule-1 (VCAM-1) are 95 and 110 kDa proteins, respectively, and both belong to the immunoglobulin superfamily. The 115 kDa protein E-selectin, which belongs to the selectin family, is expressed exclusively on endothelial cells. Recent evidence indicates that the promoter region of the gene for these adhesion molecules contains NF-κB binding sites and that these sites are essential for the expression of these genes.[40]

In the present study, we assessed the antiangiogenic activity of xanthorrhizol and curcumin on VEGF-induced proliferation, migration, and tube formation of HUVECs. We also investigated the effects of these phytochemicals on the PI3k/Akt/eNOS signaling pathway and also expression of adhesion molecules expression, which is mediated through NF-κB activation. We also performed a mouse Matrigel plug assay and a chorioallantoic membrane (CAM) assay to determine whether xanthorrhizol and curcumin are capable of blocking VEGF-induced angiogenesis *in vivo*. To our knowledge, this is the first report to show that xanthorrhizol has antiangiogenic activity both *in vitro* and *in vivo*.

II. MATERIALS AND METHODS

1. Primary cell isolation and cell culture

Freshly delivered umbilical cords were obtained from natural births and the human umbilical vein endothelial cells (HUVECs) were isolated by collagenase type Ⅱ (BD Transduction Laboratories, San Diego, CA, USA) digestion of the human umbilical vein using standard techniques. The cells were cultured in EBM-2 (Cambrex Bio Science, Walkersville, MD, USA) medium supplemented with 20% fetal bovine serum (FBS, Cambrex Bio Science, Walkersville, MD, USA) at 37℃ under a humidified 95%-5% (v/v) mixture of air and CO_2.

2. Endothelial cell proliferation assay

Cell proliferation was determined using a 3-(4,5-dimethylthiazol-2-yl) -2,5 -diphenyl tetrazoliumbromide (MTT, Sigma, St. Louis, MO, USA) assay. This assay is based on the cleavage of the yellow tetrazolium salt, MTT, by metabolically active cells to form the purple formazan crystals. HUVECs grew to confluence in a T-75 flask (NUNC, Roskilde, Denmark), and were harvested by trypsinization. The cells were seeded at a density of 5×10^3cells/well in a 96-well plate in EBM-2 containing 20% FBS. After 24 h, the cultured medium was removed completely, and treated with 200 μl of different concentrations of each compound and VEGF (20 ng/ml) in EBM-2 containing 2% FBS and incubated at 37℃. After 3 days, 20 μl of MTT reagent (5 mg/ml) was added to each well and the wells were incubated for 4 h at 37℃. The medium was removed and the MTT formazan crystals were dissolved in 200 μl of DMSO. Absorbance of each well was measured at 570 nm by an ELISA reader (Bio-Rad Benchmark, Hercules, CA, USA). Results are expressed as percentages of the OD value relative to the control cells.

3. **BrdU incorporation assay**

To study DNA synthesis, BrdU (5-bromo-2′-deoxy-uridine)-incorporation was performed with a BrdU-ELISA-kit (5-bromo-2′-deoxy-uridine Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany) following vendors protocol. Briefly, the cells were seeded at a density of 5×10^3cells/well in a 96-well plate in EBM-2 containing 20% FBS. After 24 h, the cultured medium was removed completely, and treated with 200 μl of different concentrations of each compound and VEGF (20 ng/ml) in EBM-2 containing 2% FBS and incubated at 37℃. At the end of treatment, 10 μl BrdU labeling solution (final conc. 10 μM BrdU) was added to each well and incubated for 4 h at 37℃ followed by fixation and incubation with anti-BrdU peroxidase conjugate for an additional 30 min at 37℃. Finally, after substrate reaction, color intensity was measured at 570nm by an ELISA reader. Results are expressed as percentages of the OD value relative to the control cells.

4. **Endothelial 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 μm pore size). Briefly, the lower surface of the filter was coated with 10 μg of gelatin. Fresh medium (2% FBS) containing VEGF was placed in the lower wells. Cells were trypsinized and suspended at a final concentration of 1×10^6cells/ml in EBM-2 containing 2% FBS. Various concentrations of each compound and VEGF (20 ng/ml) were given to the cells. Then, 100 μl of the cell suspension was loaded into each of the upper wells. The chamber was incubated at 37℃ for 7 h. The cells were fixed with methanol and stained with hematoxylin/eosin. The nonmigrating cells on the upper surface of the filter were removed by wiping 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 ×200 magnification. Each sample was assayed in triplicate, and the experiment was repeated three times independently.

5. Endothelial cell tube formation assay

The HUVECs at subconfluence were switched to serum-free EBM-2 medium overnight. Then, 250 μl 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℃. The starved HUVECs were trypsinized and suspended in EBM-2 containing 2% FBS. The cells were mixed with various concentrations of each compound and VEGF (20 ng/ml) and seeded to the Matrigel pretreated 24-well plate at a density of 1×10^5cells/well. After 7 h of incubation at 37℃, the morphological changes in the cells were observed under a microscope and photographed at × 40 magnification.

6. Transfection and dual luciferase assays

Transient transfections of HUVECs were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Cells were transfected with 4 μg of the firefly luciferase reporter plasmids containing the human NF-κB promoter sequence (generous gifts from prof. Sang-kook Lee, Ewha Womans University). HUVECs were cotransfected with 0.4 μg of the *Renilla* luciferase control vector to normalize for transfection efficiency. After the transfections, cultures were maintained in normal growth medium for 24 h and then exposed to different concentrations of each compound and VEGF (20 ng/ml) for additional 16 h EBM-2 media containing 10 % FBS. All reactions of firefly and *Renilla* luciferase were performed using the

Dual-Luciferase Reporter Assay System (Promega, Madison, USA). Briefly, the cells were washed and lysed with Passive Lysis Buffer. Cell lysates were mixed with Luciferase Assay Reagent Ⅱ, and the firefly luminescence was measured using a luminometer with dual automatic injector. The samples were then mixed with the Stop & Glo reagent, and the *Renilla* luciferase activity was measured as an internal control.

7. Chorioallantoic membrane (CAM) assay

Fertilized chicken eggs (Pulmuone Co, Seoul, Korea) were kept in a humidified incubator at 37℃ for 3 days. On day 3, about 2 ml of egg albumin was removed with a hypodermic needle through a small hole drilled at the narrow end of the egg, allowing the CAM and yolk sac to drop away from the shell membrane. The shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away. When the chicken embryo was 4 days old, each of the compound loaded thermanox coverslips were inverted and applied to the CAM surface through the window and the window was closed. Two days later, an appropriate volume of a 10% fat emulsion was injected into the CAM so that the vascular network of the CAM stood out against the white background of lipid. The CAM was inspected for changes in the density of blood vessels and photographed using image pro analysis software. The experiment was repeated three times and 10 eggs were used each time.

8. *In vivo* mouse Matrigel plug assay

The Matrigel was thawed overnight at 4℃. Before the injection into C57BL/6 mice (7 week-old male, Orient Co. Seoul, Korea) it was mixed with 20 units of heparin

(Sigma, St. Louis, MO, USA), 100 ng/ml VEGF, and either 10 or 20 μM of xanthorrhizol and curcumin. The Matrigel mixture (600 μl) was injected subcutaneously using a 21-gauge needle. The injected Matrigel rapidly formed a single, solid gel plug. We also evaluated the effect of the oral administration of each compound. Mice received xanthorrhizol and curcumin (0.02 mg/kg) daily, by oral route, for 6 days after Matrigel implantation. After 7 days, the skin of the mouse was easily pulled back to expose the Matrigel plug, which remained intact. After being photographed, the plugs from all groups were collected and weighed. Samples were minced and diluted in water to measure the hemoglobin contents. To quantify the formation of a functional vasculature in the Matrigel plug, the amount of hemoglobin was measured using Drabkin's reagent kit 525 (Sigma, St. Louis, MO, USA). The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel. Results from the *in vivo* experiment are expressed as mean g/dl of hemoglobin ± SE. Statistical analyses of the data were performed using the paired Student's *t*-test, with $p < 0.05$ considered statistically significant.

9. Western blot analysis

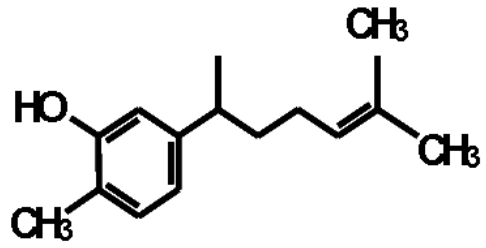
The confluent HUVECs were placed in serum-free medium overnight prior to treatment. After treatment with various agents and conditions, cells were washed with cold phosphate buffered saline (PBS, GIBCO, Grand Island, NY, USA), and lysis buffer (10 mmol/L Tris-HCl, pH7.4, with 1% NP-40, 0.1% sodium deoxycholate) containing proteinase inhibitors (Complete, Roche, Mannheim, Germany) was added followed by centrifugation at 15,000g for 20 min at 4℃. Supernatants were separated and used as whole cell extracts. Total protein concentrations were determined using bovine serum albumin as a standard. Samples (40 μg protein) were separated on an 8-10% gradient polyacrylamide gel. For NF-κB, nuclear extracts were prepared with

lysis buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and buffer C (20 mM HEPES, 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF). Fifteen micrograms of nuclear protein was used in each experiment and transferred to polyvinylidene fluoride (PVDF) membrane (Amersham, Arlington Heights, IL, USA). The membranes were incubated with Tween-TBS containing 5% nonfat dry milk at room temperature for 2 h to block nonspecific antibody binding. Membranes were then incubated with an anti-Akt antibody, an anti-phospho-Akt antibody (Cell Signaling, Beverly, MA, USA), an anti-eNOS antibody, an anti-phospho-eNOS antibody (BD Transduction Laboratories, San Diego, CA, USA), an anti-ICAM-1 antibody, an anti-VCAM-1 antibody, an anti-E-selectin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), an anti-p65 antibody, an anti-p50 antibody (Cell Signaling, Beverly, MA, USA), or an anti-β-actin antibody (Sigma, St. Louis, MO, USA) overnight at 4℃. The membranes were then incubated with an appropriate peroxidase-conjugated secondary antibody and identified using a chemiluminescence detection kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

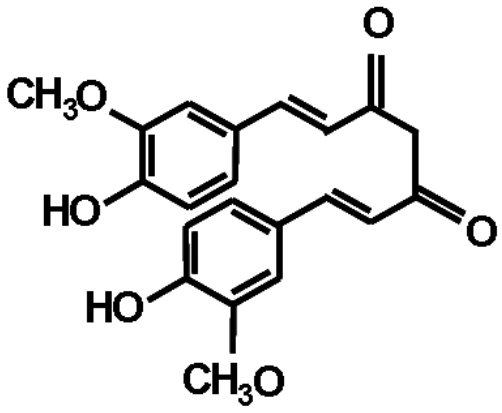
III. RESULTS

1. Xanthorrhizol and curcumin inhibit VEGF-induced proliferation of HUVECs

The inhibitory effects of xanthorrhizol and curcumin on VEGF-induced proliferation of HUVECs were evaluated to determine the antiangiogenic activity of these phytochemicals *in vitro*. Both xanthorrhizol and curcumin inhibited VEGF-induced HUVECs proliferation in a dose-dependent manner, with half maximal inhibition at 10 μM and 5 μM, respectively (Figure 2A). Quantification of BrdU incorporation during DNA synthesis in proliferating cells was an index of cell viability. At 1-20 μM doses, xanthorrhizol and curcumin treatment of HUVECs resulted in 24-50% and 21-42% inhibition in BrdU incorporation, respectively, suggesting that inhibition of DNA synthesis might be a significant contributor in xanthorrhizol and curcumin-caused cell proliferation inhibition in HUVECs (Figure 2B). This inhibitory effect was not due to cytotoxicity of xanthorrhizol in endothelialcell, because xanthorrhizol had no effect on normal growth of HUVECs devoid of VEGF stimulation up to 20 μM (showed over 80% of viability) (Figure 2C).



xanthorrhizol



curcumin

Figure 1. **Chemical structure of** x**anthorrhizol and curcumin**

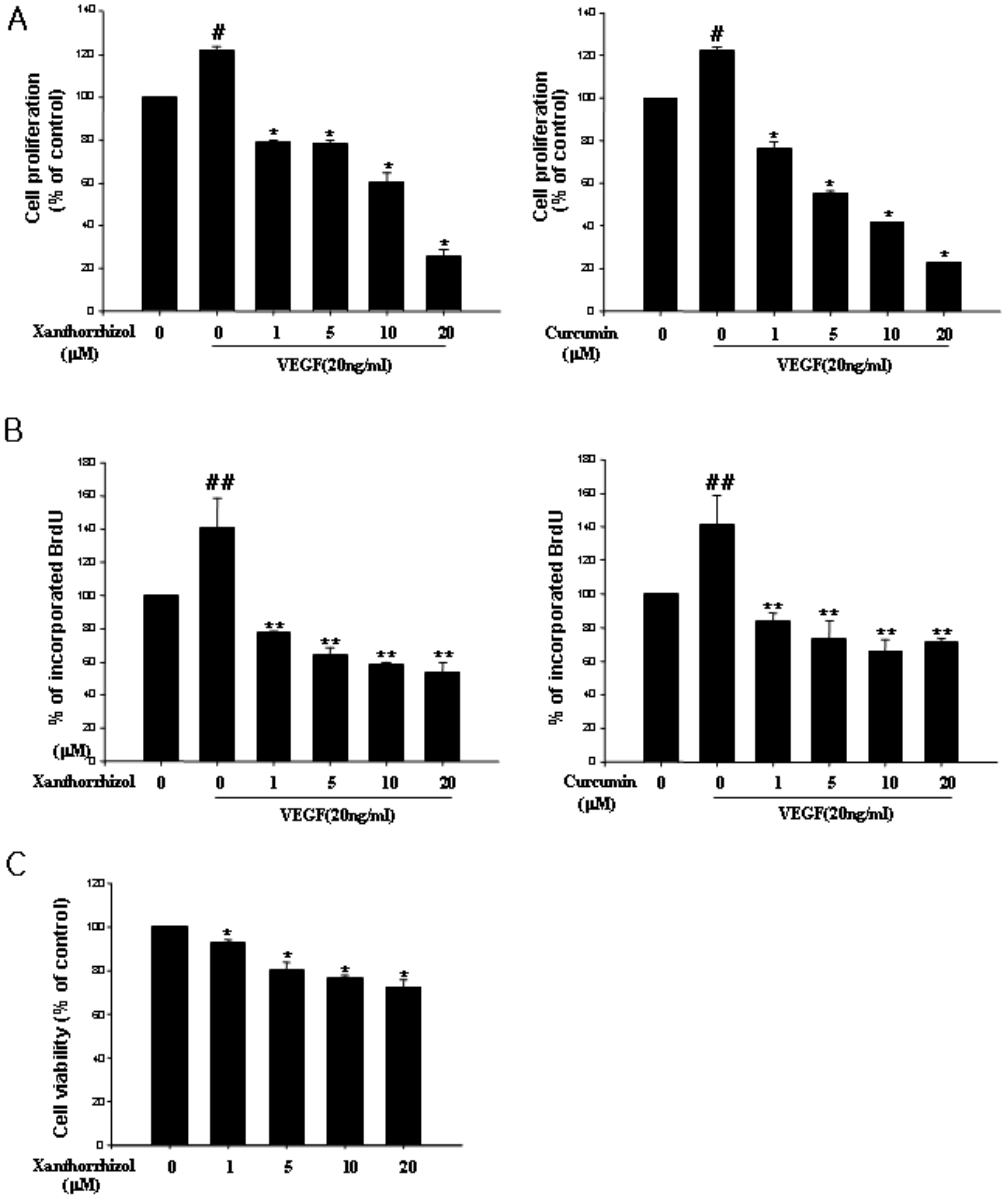

A
Cell proliferation (% of control)
Xanthorrhizol (μM)
VEGF(20ng/ml)
Curcumin (μM)
B
% of incorporated BrdU
C
Cell viability (% of control)

Figure 2. The effects of xanthorrhizol and curcumin on vascular endothelial growth factor (VEGF)-induced proliferation and DNA synthesis of HUVECs. *X*anthorrhizol and curcumin inhibit VEGF-induced proliferation. HUVECs were treated with various concentrations (1-20 μM) of xanthorrhizol and curcumin in the presence of VEGF (20 ng/ml). After 72 h incubation, the cell viability was measured by an MTT assay (A). Xanthorrhizol and curcumin inhibit DNA synthesis. 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 multi-well microplate reader at 405 nm as detailed in Materials and methods (B). Treatment of xanthorrhizol on HUVECs without VEGF. HUVECs were treated with 1-20 μM of in the absence of VEGF. After 72 h incubation, the cell viability was measured by an MTT assay (C). The data are presented as mean±SE; #, p <0.001, ##, p <0.01 versus VEGF-non treated group and *, p <0.001, **, p <0.005 versus VEGF alone; bars

2. Xanthorrhizol and curcumin inhibit VEGF-induced migration and tube formation of HUVECs

Migrating endothelial cells were investigated because they are an important in angiogenesis. The effects of xanthorrhizol and curcumin on the chemotactic motility of HUVECs were measured using a Transwell plate. VEGF (20ng/ml) significantly increased cell migration, but this effect was blocked by xanthorrhizol and curcumin dose-dependently (Figure 3).

Next, the effects of xanthorrhizol and curcumin on the morphological differentiation of HUVECs were investigated using a two-dimensional Matrigel. When HUVECs were placed on a growth factor-reduced Matrigel in the presence of VEGF (20 ng/ml), VEGF led to the formation of elongated and robust tube-like structures. Both compounds effectively abrogated the width and the length of endothelial tubes induced by VEGF in a concentration-dependent manner (Figure 4). Treatment with 10 μM of xanthorrhizol and curcumin showed incomplete tube formation in the VEGF-treated HUVECs, whereas treatment with 20 μM of each compound inhibited tube formation completely.

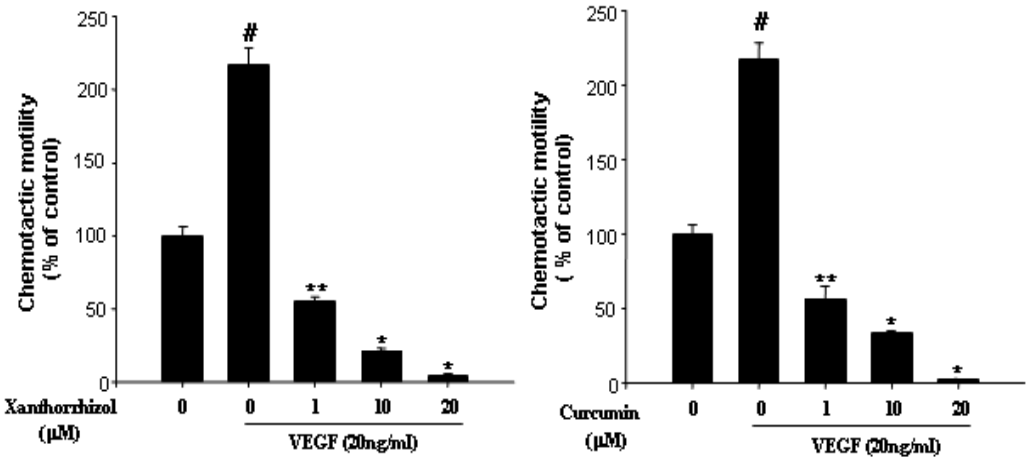


Figure 3. Xanthorrhizol and curcumin inhibit VEGF-induced migration of HUVECs. Chemomigration of the HUVECs was performed using a 12-well Transwell culture chamber with 8.0 μm-pore-polycarbonate filter inserts. The lower surface of the filter was coated with 10 μl gelatin (1 g/ml). HUVECs were placed in the upper part of the filter treated with various concentrations of xanthorrhizol and curcumin in the presence of VEGF (20 ng/ml). After 7 h incubation, chemotaxis was quantified by counting the cell that migrated to the lower side of the filter with optical microscope at ×200 magnification. The data are presented as mean±SE; #, $p<0.005$, versus VEGF-non treated group and *, $p<0.005$, **, $p<0.01$ versus VEGF alone; bars

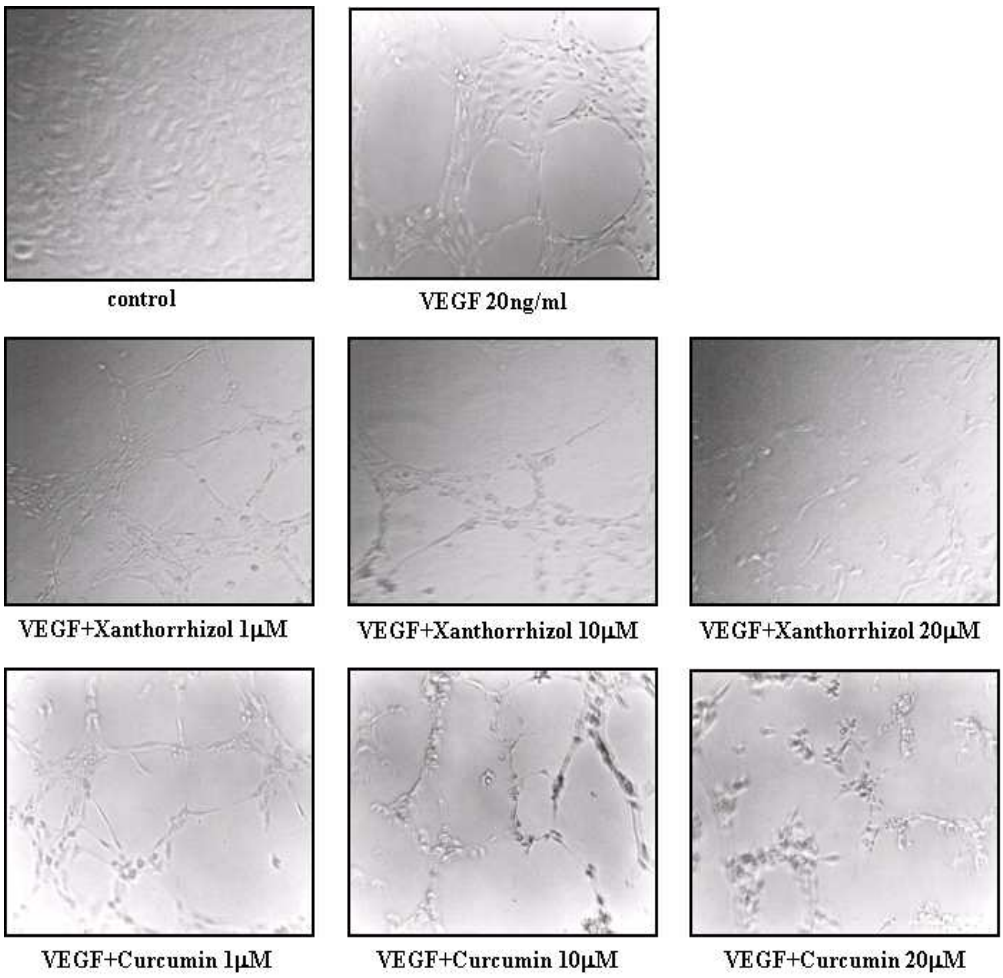


Figure 4. Xanthorrhizol and curcumin inhibit VEGF-induced tube formation of HUVECs. HUVECs were collected and replated on Matrigel-coated 24-well plates at a density of 2×10^5cells/well and then incubated in the presence of various concentrations of xanthorrhizol and curcumin with VEGF (20 ng/ml). After 8 h, microphotographs were taken (×40).

3. The up-regulation of the phosphorylation of Akt and eNOS expression by VEGF in HUVECs

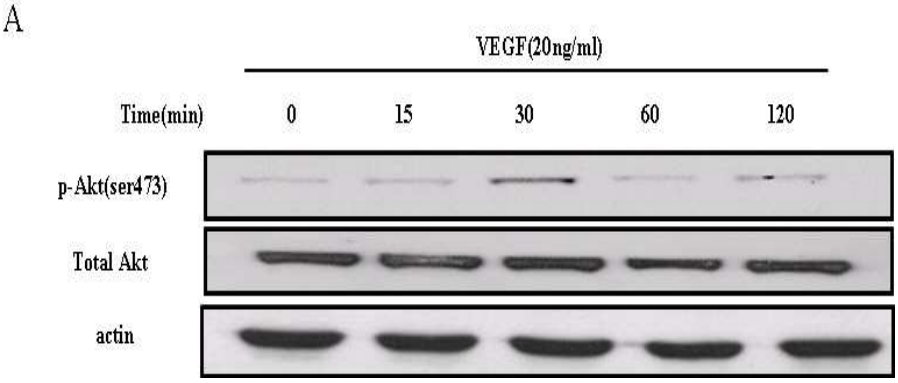
To test whether VEGF increases Akt kinase and eNOS activity in endothelial cells, HUVECs were stimulated with VEGF for various durations, and the phosphorylation of Akt and eNOS was assessed by a Western blot assay using anti-phospho-Akt and anti-phospho-eNOS antibodies. As shown in Figure 5A and Figure 6A, VEGF increased Akt and eNOS phosphorylation significantly compared to the phosphorylation observed in unstimulated HUVECs at 30 min.

4. The effects of xanthorrhizol and curcumin on VEGF-induced phosphorylation of Akt and eNOS expression via phosphatidylinositol 3-kinase (PI3k) in HUVECs

It has been established that the Akt/NO pathway is critical for VEGF-induced endothelial cell migration, proliferation, and tube formation *in vitro*. VEGF induced phosphorylation of Akt (Ser473) and endothelial NO synthase (eNOS:Ser1177) plays a key role in VEGF-stimulated angiogenesis. Therefore, the effects of xanthorrhizol and curcumin on VEGF-induced Akt and eNOS phosphorylation were evaluated using antibodies directed against the phosphorylated forms of Akt and eNOS, respectively. Because VEGF induces a maximal effect on the expression of these phosphorylated forms at 30 min, we examined the effects of each compound at this time point. Pretreatment of cells with xanthorrhizol and curcumin for 1 h seemed to be more effective than cotreatment with both compounds. In the case of pretreatment, both xanthorrhizol and curcumin significantly diminished VEGF-induced Akt and eNOS phosphorylation in a dose-dependent manner without any change in total Akt and eNOS protein levels (Figure 5B, Figure 6B). At a dose of 20 μM of xanthorrhizol

and curcumin, phosphorylated forms of Akt and eNOS were abolished completely. Curcumin also showed a slight decrease in total Akt and eNOS levels but xanthorrhizol did not. HUVECs were also treated with specific PI3k inhibitor, wortmannin, to identify the involvement of PI3k with Akt and eNOS. The phosphorylation of Akt and eNOS were also blocked by pretreatment with wortmannin (Figure 5B, Figure 6B).

A


VEGF(20ng/ml)
Time(min)
0
15
30
60
120
p-Akt(ser473)
Total Akt
actin

B

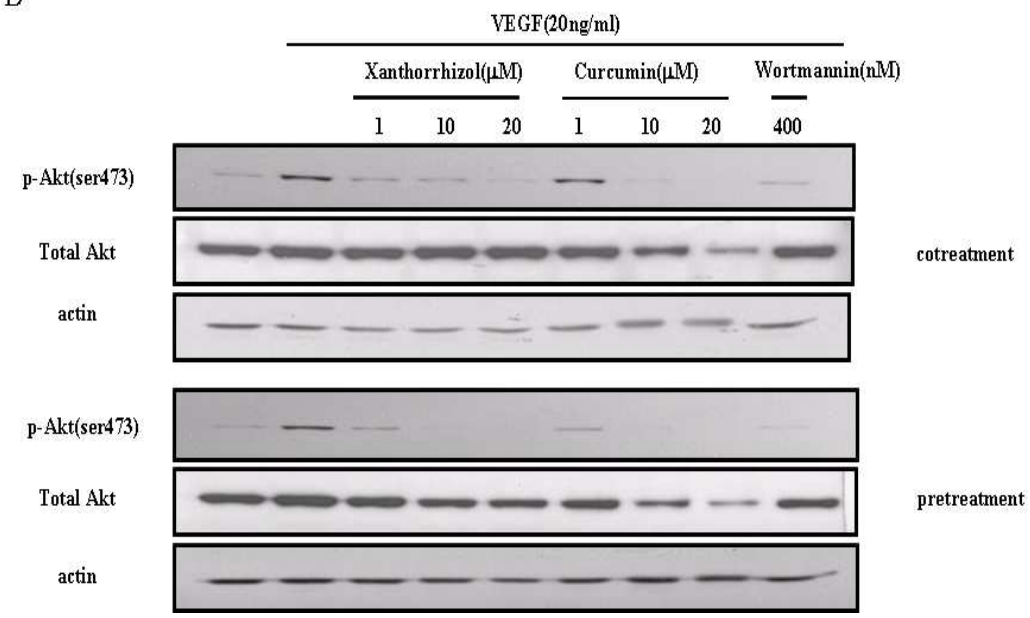
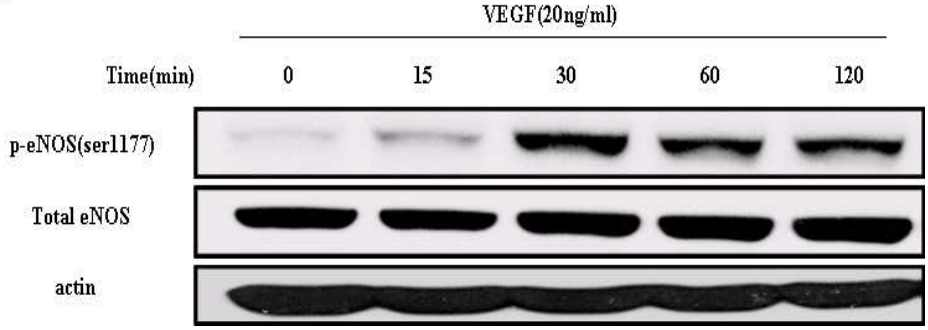

VEGF(20ng/ml)
Xanthorrhizol(μM)
Curcumin(μM)
Wortmannin(nM)
1
10
20
1
10
20
400
p-Akt(ser473)
Total Akt
actin
cotreatment
p-Akt(ser473)
Total Akt
actin
pretreatment

Figure 5. Xanthorrhizol and curcumin downregulate phosphorylation of Akt induced by VEGF in HUVECs. Phospho-Akt was maximally expressed at 30 min after VEGF stimulation and was determined by Western blot analysis (A). Akt phosphorylation at the Ser473 was measured after HUVECs were cotreated or pretreated with various concentrations (1, 10, 20 μM) of xanthorrhizol and curcumin in the presence of VEGF (20 ng/ml) for 30 min. HUVECs were also treated for 30 min with the PI3k specific inhibitor, wortmannin (400 nM) (B). Total cell lysate 40 μg from each sample was resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride transfer membrane, and probed with anti-phospho-Akt antibody. The same blots were stripped and reprobed with antibodies to Akt (total Akt) and to actin to demonstrate equal protein loading. Specific bands were visualized using the enhanced chemiluminescence detection kit (ECL) system.

A


VEGF(20ng/ml)
Time(min)
0
15
30
60
120
p-eNOS(ser1177)
Total eNOS
actin

B

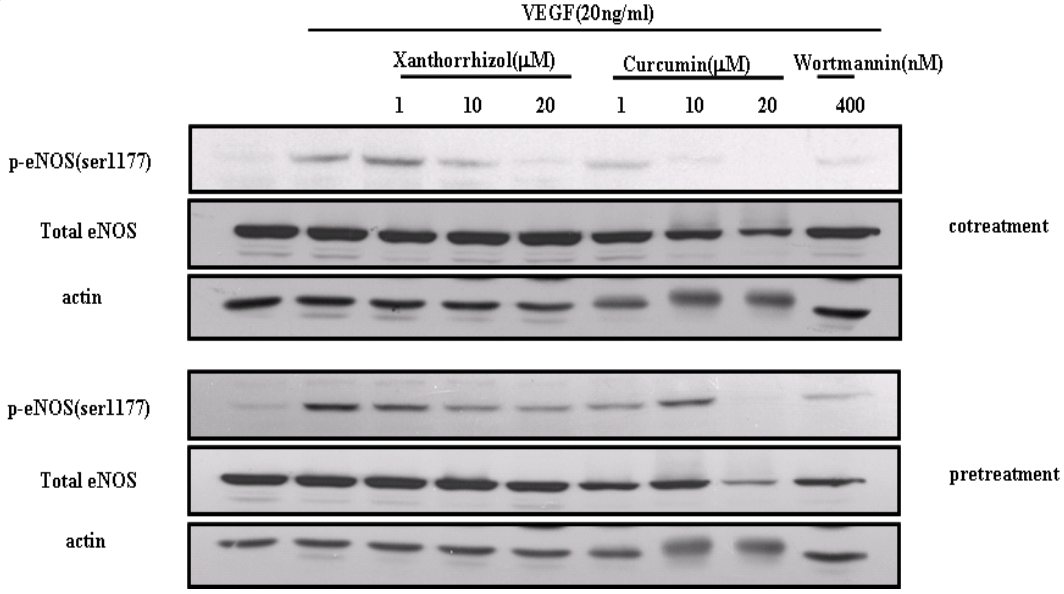

VEGF(20ng/ml)
Xanthorrhizol(μM)
Curcumin(μM)
Wortmannin(nM)
1
10
20
1
10
20
400
p-eNOS(ser1177)
Total eNOS
actin
cotreatment
p-eNOS(ser1177)
Total eNOS
actin
pretreatment

Figure 6. Xanthorrhizol and curcumin downregulate phosphorylation of eNOS induced by VEGF in HUVECs. Phospho-eNOS was maximally expressed at 30 min after VEGF stimulation (A). Phosphorylation at the Ser1177 position was measured after HUVECs were cotreated or pretreated with various concentrations (1, 10, 20 μM) of xanthorrhizol and curcumin in the presence of VEGF (20 ng/ml) for 30 min. HUVECs were also treated for 30 min with the PI3k specific inhibitor, wortmannin (400 nM) (B). Total cell lysate 40 μg from each sample was resolved by 8% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride transfer membrane, and probed with anti-phospho-eNOS antibody. The same blots were stripped and reprobed with antibodies to eNOS (total eNOS) and to actin to demonstrate equal protein loading. Specific bands were visualized using the ECL system.

5. The up-regulation of adhesion molecules expression by VEGF in HUVECs

HUVECs were stimulated with VEGF for up to 24 h to elucidate the effects of VEGF on ICAM-1, VCAM-1, and E-selectin expression. The addition of 20 ng/ml VEGF increased the expression of ICAM-1, VCAM-1, and E-selectin and produced a maximal effect at 6 h (Figure 7).

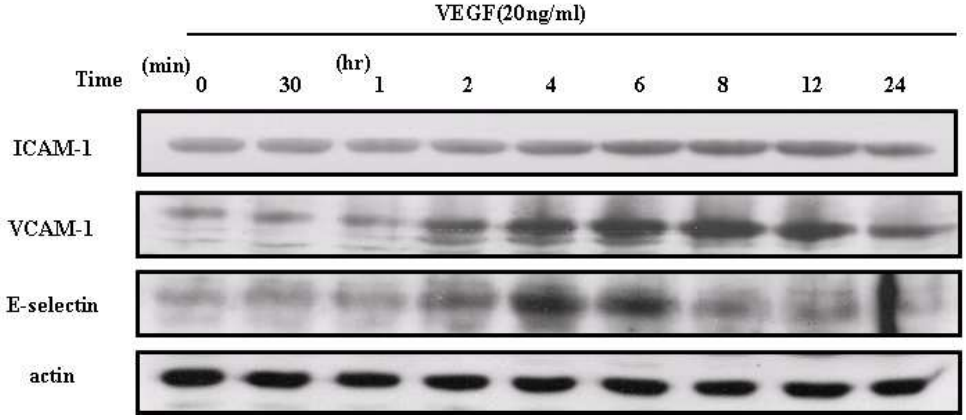


Figure 7. The effects of VEGF on the time course of ICAM-1 and VCAM-1, and E-selectin expression in HUVECs. HUVECs were incubated with 20 ng/ml VEGF for the indicated times. Each lane contains 40 μg of cell lysate protein. The Western blot was probed with an anti-ICAM-1 antibody or an VCAM-1 antibody, or an E-selectin antibody. The same blots were stripped and reprobed with anti-actin antibody to verify equal loading of protein in each lane.

6. The effects of xanthorrhizol and curcumin on VEGF-induced adhesion molecules expression in HUVECs

The expression of CAMs on the surface of endothelial cells is required for cell-cell, cell-ECM, and cell-leukocyte interactions. The expression of ICAM-1, VCAM-1, and E-selectin were examined to determine the inhibitory effects of xanthorrhizol and curcumin on VEGF-induced adhesion molecule expression upon VEGF stimulation. Because VEGF produces a maximum effect on expression of these adhesion molecules at 6 h, we examined the effects of each compound at this time point.

Previous data revealed that pretreatment with xanthorrhizol and curcumin was more effective than cotreatment. Based on this information, we also used xanthorrhizol and curcumin pretreatment for the *in vitro* experiment. Treatment with curcumin 1 h prior to VEGF-stimulation prevented upregulation of ICAM-1. Xanthorrhizol, however, failed to significantly modulate the VEGF-induced expression of ICAM-1 at any of the concentrations used. Instead, xanthorrhizol upregulated the expression of ICAM-1, especially at a dose of 10 μM (Figure 8A). As shown Figure 8B, xanthorrhizol and curcumin, at doses ranging from 1 to 20 μM, induced significant dose-dependent inhibition of VCAM-1 protein expression. At a dose of 20 μM, both compounds completely abolished the VEGF-induced expression of VCAM-1. Xanthorrhizol and curcumin also inhibited E-selectin expression in a dose-dependent manner (Figure 8C). Xanthorrhizol seemed to be more effective than curcumin. Unexpectedly, the PI3k kinase inhibitor, wortmannin (400 nM), enhanced VEGF-stimulated ICAM-1, VCAM-1, and E-selectin expression.

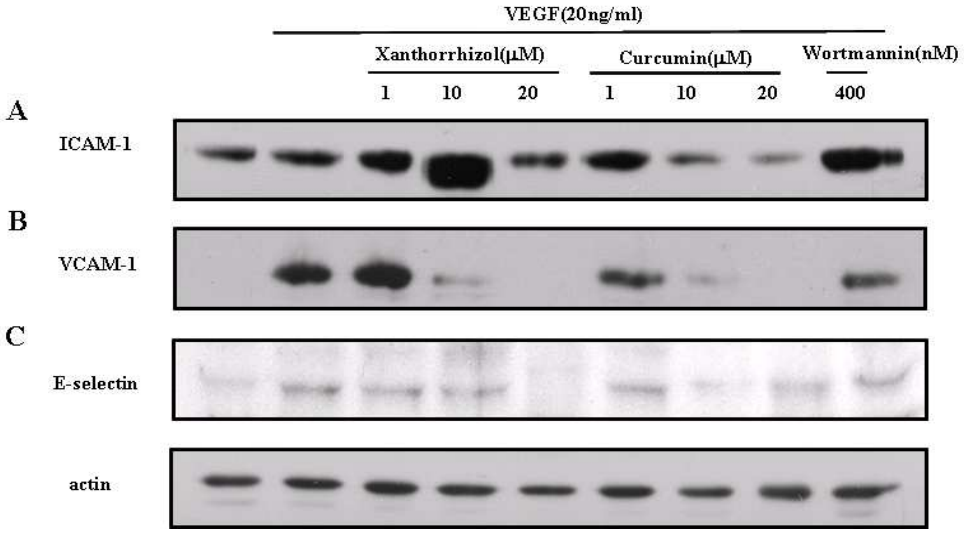


Figure 8. **Xanthorrhizol and curcumin inhibit** VEGF **induced** ICAM-1 **and** VCAM-1, **and E-selectin** expression **in** HUVECs. HUVECs **were pre-incubated for an hour with the indicated doses of** xanthorrhizol **and curcumin, followed by stimulation with** VEGF **(20 ng/ml) for 6 h.** Expression **of** ICAM-1 **(A) and** VCAM-1 **(B), and E-selectin (C) in** HUVECs **after pre-incubation with** xanthorrhizol **and curcumin was measured by Western blot analysis.**

7. Inhibitors changed the VEGF-induced expression of ICAM-1, VCAM-1, and E-selectin proteins in HUVECs

The PI3k inhibitor, wortmannin (400 nM), the nitric oxide synthase (NOS) inhibitor, L-NAME (5 mM), the NF-κB inhibitor, parthenolide (5 μM), and the PKC inhibitor, chelerythrine chloride (5 μM) were added to the HUVECs 1 h prior to VEGF-stimulation to investigate the major mechanism responsible for the induction of adhesion molecules by VEGF. Consistent with previous results, wortmannin enhanced VEGF-induced expression of ICAM-1, VCAM-1, and E-selectin. Parthenolide suppressed VEGF-induced expression of ICAM-1, VCAM-1, and E-selectin whereas L-NAME did not induce any effect on adhesion molecule expression. The treatment using the NF-κB inhibitor, parthenolide, completely eliminated the VEGF-induced expression of VCAM-1 and E-selectin compared to ICAM-1. The PKC inhibitor, chelerythrine chloride, also suppressed the VEGF-induced expression of adhesion molecules, but not ICAM-1 (Figure 9). These results suggest that VEGF-stimulated expression of ICAM-1, VCAM-1 and E-selectin may be mediated mainly through the activation of NF-κB, along with PI3k-mediated suppression. The induction of NO may not be involved in the regulation of ICAM-1, VCAM-1, and E-selectin.

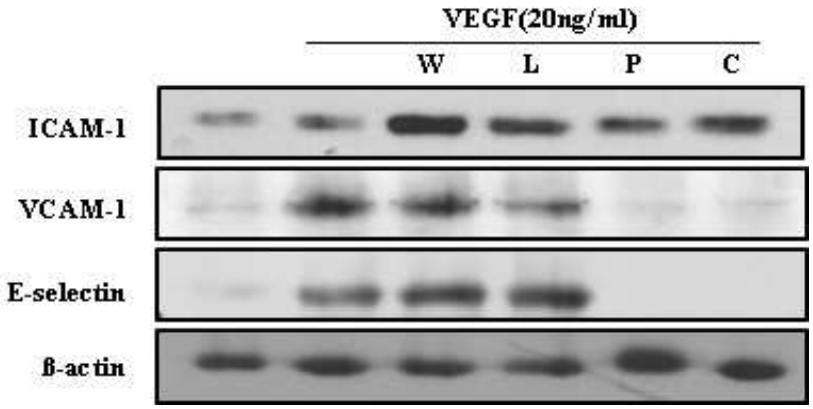
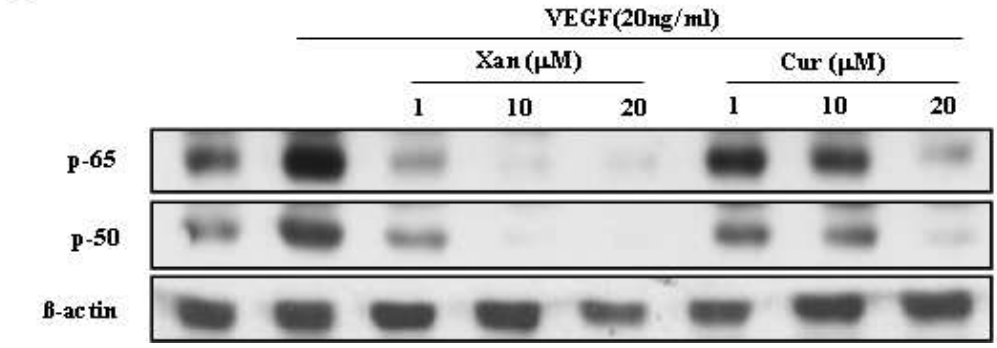


Figure 9. Expression of adhesion molecules in VEGF-induced HUVECs pre-treated with various inhibitors. HUVECs were pre-incubated for an hour with the PI3k inhibitor, wortmannin (W, 400 nM), the nitric oxide synthase (NOS) inhibitor, L-NAME (L, 5 mM), the NF-κB inhibitor, parthenolide (P, 5 μM), and the PKC inhibitor, chelerythrine chloride (C, 5 μM), followed by stimulation with VEGF (20 ng/ml) for 6 h. Expression of ICAM-1 and VCAM-1, and E-selectin in HUVECs was measured by Western blot analysis.

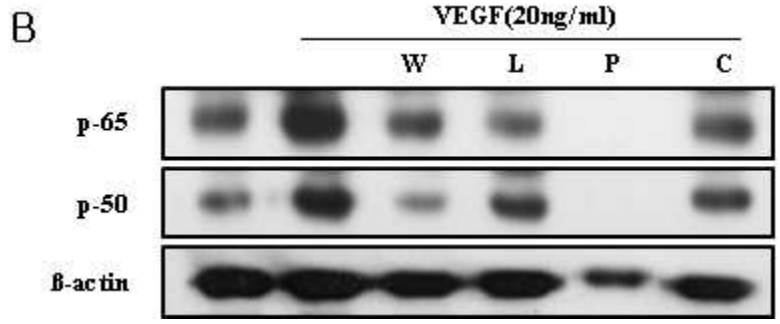
8. VEGF-induced expression of ICAM-1, VCAM-1, and E-selectin was correlated with NF-κB activity

Because the expression of adhesion molecules was mainly regulated by NF-κB, we examined NF-κB activity in HUVECs treated with VEGF in the absence or presence of xanthorrhizol and curcumin. We analyzed the p65 and p50 subunits of NF-κB in the nuclear extract by immunoblotting. Xanthorrhizol and curcumin decreased nuclear levels of both p50 and p65. Xanthorrhizol exhibited a stronger NF-κB inhibitory effects than did curcumin at the same doses. (Figure 10A). We also used the PI3k inhibitor, wortmannin, the nitric oxide synthase (NOS) inhibitor, L-NAME, the NF-κB inhibitor, parthenolide, and the PKC inhibitor, chelerythrine chloride. Wortmannin unexpectedly decreased VEGF-induced p65 and p50 subunits whereas it enhanced the VEGF-induced expression of ICAM-1, VCAM-1, and E-selectin. The NOS inhibitor, L-NAME and the PKC inhibitor, chelerythrine chloride decreased levels of the p65 and p50 to basal levels, and the NF-κB inhibitor, parthenolide, completely blocked nuclear translocation of both subunits (Figure 10B). These observations suggest that the inhibitory effects of adhesion molecules on xanthorrhizol and curcumin could be mainly dependent on the NF-κB pathway. To further clarify the effects of xanthorrhizol and curcumin on the VEGF-mediated NF-κB gene expression, transcriptional activities of the human NF-κB promoter were measured. As indicated in Figure 10C, VEGF significantly increased NF-κB promoter activity by approximately 4.6-fold. However, treatment with xanthorrhizol and curcumin abolished the VEGF-induced transcriptional activation of the NF-κB promoter construct dose-dependently.

A


VEGF(20ng/ml)
Xan (μM)
Cur (μM)
1
10
20
1
10
20
p-65
p-50
ß-actin

B


VEGF(20ng/ml)
W
L
P
C
p-65
p-50
ß-actin

C

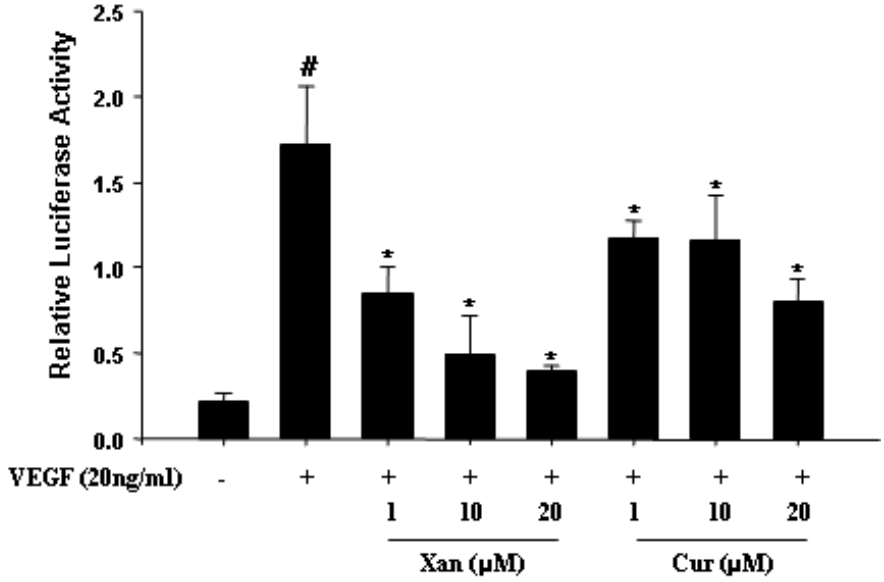
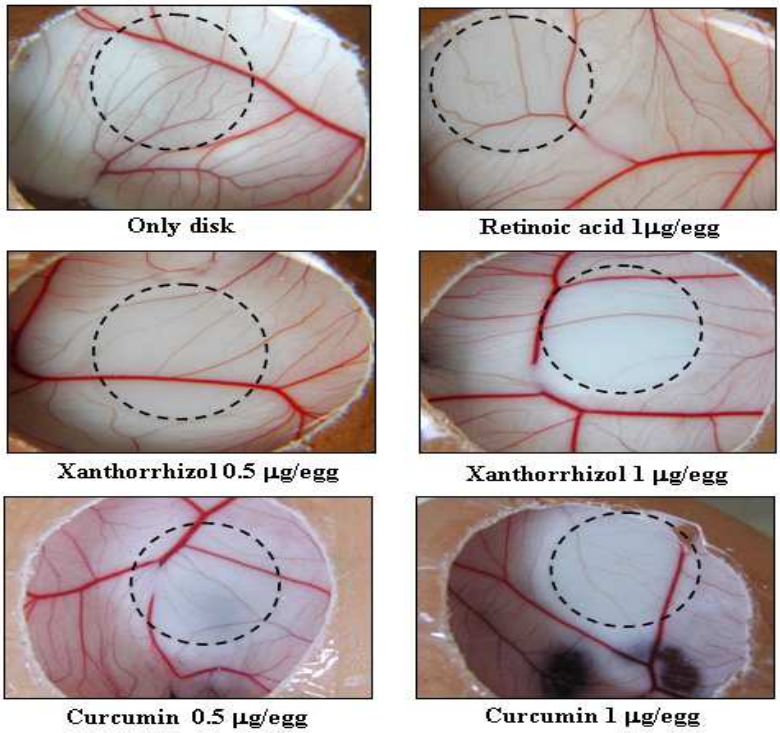

Relative Luciferase Activity
2.5
2.0
1.5
1.0
0.5
0.0
#
*
VEGF (20ng/ml) - + + + + + + +
1 10 20 1 10 20
Xan (μM)
Cur (μM)

Figure 10. Activation of NF-κB by VEGF and effects of xanthorrhizol and curcumin, and inhibitors. HUVECs were pre-incubated for an hour with the indicated doses of xanthorrhizol and curcumin, followed by stimulation with VEGF (20 ng/ml) for 6 h (A). HUVECs were also pre-incubated for an hour with the PI3k inhibitor, wortmannin (W, 400 nM), the nitric oxide synthase (NOS) inhibitor, L-NAME (L, 5 mM), the NF-κB inhibitor, parthenolide (P, 5 μM), and the PKC inhibitor, chelerythrine chloride (C, 5 μM), and then treated with VEGF (20 ng/ml) for 6 h (B). Nuclear extract was analyzed for nuclear translocation of NF-κB members. Nuclear extract 15 μg from each sample was resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride transfer membrane, and probed with anti-p65 antibody. The same blots were stripped and reprobed with anti-p50 antibody and to actin to demonstrate equal protein loading. Specific bands were visualized using the ECL system. Xanthorrhizol and curcumin attenuate transcriptional activation of the human NF-κB promoter in VEGF-treated HUVECs (C). HUVECs were transfected with the firefly luciferase reporter plasmids containing the human NF-κB promoter sequences, treated with xanthorrhizol and curcumin at concentrations of 1-20 μM in a presence of VEGF (20ng/ml) for 16 h. NF-κB promoter activity was analyzed by dual luciferase assay as described in Materials and Methods. The data are presented as mean±SE; #, $p<0.01$ versus VEGF-non treated group and *, $p<0.05$ versus VEGF alone; bars

9. The effects of xanthorrhizol and curcumin on the chorioallantoic membrane (CAM) assay

The ability of xanthorrhizol and curcumin to inhibit *in vivo* angiogenesis was then examined using the chick chorioallantoic membrane (CAM) assay. Retinoic acid, which is known to have anti-angiogenic activity, was used as a positive control compound. Xanthorrhizol and curcumin caused avascular zones, reflecting anti-angiogenic activity on the treated CAMs, while treatment with an empty coverslip (control) did not produce an effective response. The dose-response relationship for the inhibition of embryonic angiogenesis is shown in Figure 11A. At the dose of 1 μg/egg, xanthorrhizol and curcumin inhibited the vessels formation to 29% and 19.5%, respectively (Figure 11B). No signs of thrombosis or hemorrhage were detected in the dose range used. These results clearly indicate that xanthorrhizol and curcumin are potent antiangiogenic molecules *in vivo*.

A


Only disk
Retinoic acid 1μg/egg
Xanthorrhizol 0.5 μg/egg
Xanthorrhizol 1 μg/egg
Curcumin 0.5 μg/egg
Curcumin 1 μg/egg

B

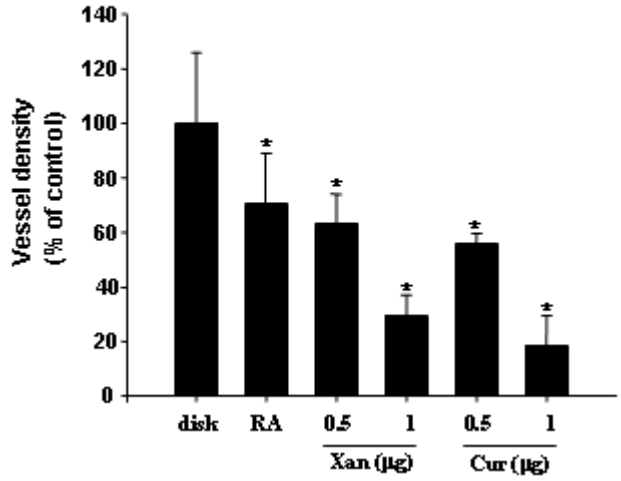

Vessel density
(% of control)
140
120
100
80
60
40
20
0
*
disk
RA
0.5
1
Xan (μg)
0.5
1
Cur (μg)

Figure 11. Effects of xanthorrhizol and curcumin on embryonic angiogenesis in CAM. Fertilized chick eggs were kept in a humidified incubator at 37℃. At the stage of 4.5-day embryo, xanthorrhizol, curcumin or retinoic acid (RA)-loaded thermanox coverslip was applied to the CAM surface. Two days later, fat emulsion (10%) was injected into chorioallantois to make the vascular network clear. The chorioallantois was observed under a microscope. Retinoic acid was loaded as a positive control. Dotted circles denote the area covered by the coverslip (A). Quantification of newly formed blood vessels density (B). The data are presented as mean±SE; *, <0.05 versus disk alone; bars

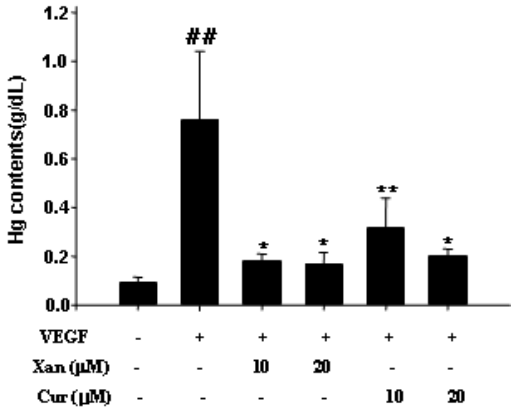
10. The effects of xanthorrhizol and curcumin on VEGF-induced vessel formation in the *in vivo* Matrigel plug assay

To determine whether xanthorrhizol and curcumin are capable of blocking VEGF-induced angiogenesis *in vivo*, an established *in vivo* angiogenesis model, the mouse Matrigel plug assay, was performed. Matrigel containing VEGF (100 ng) with or without xanthorrhizol and curcumin (10 or 20 μM, respectively) were subcutaneously injected into C57BL/6 mice. Seven days later, the Matrigel plugs that formed in the mice were excised and photographed. Plugs with VEGF alone appeared dark-red in color, which indicates the formation of a functional vasculature inside the Matrigel and blood circulation in newly formed vessels by angiogenesis induced by VEGF. In contrast, plugs with Matrigel alone and mixed xanthorrhizol or curcumin were pale in color indicating the absence of or marginal blood vessel formation (Figure 12A). The hemoglobin content inside the Matrigel plugs was measured to quantify the angiogenesis inhibited by xanthorrhizol and curcumin. The hemoglobin content in VEGF-treated was increased nearly 800%, and xanthorrhizol and curcumin markedly inhibited the hemoglobin content quantity to 25% and 40% at 10 μM, respectively (Figure 12B). We also evaluated the effects of the oral administration of both compounds. Daily treatment of xanthorrhizol and curcumin (0.02 mg/kg, p.o.) also significantly reduced the angiogenic response evoked by VEGF without weight loss (Figure 12C). These results indicate that xanthorrhizol and curcumin are capable of inhibiting VEGF-induced neovessel formation *in vivo* model system and that they are effective as an oral agent.

A


control
VEGF (100ng/ml)
VEGF + Xan 10μM
VEGF + Xan 20μM
VEGF + Cur 10μM
VEGF + Cur 20μM

B


Hg contents(g/dL)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
##
*
*
**
*
VEGF - + + + + +
Xan (μM) - - 10 20 - -
Cur (μM) - - - - 10 20

C

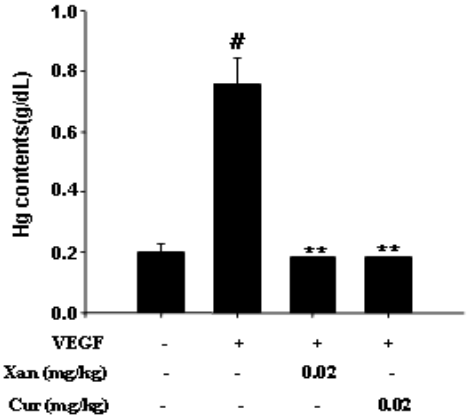

Hg contents(g/dL)
1.0
0.8
0.6
0.4
0.2
0.0
#
**
**
VEGF - + + +
Xan (mg/kg) - - 0.02 -
Cur (mg/kg) - - - 0.02

Figure 12. Xanthorrhizol and curcumin inhibit VEGF-induced angiogenesis *in vivo*. C57BL/6 mice were injected with 600 μl of Matrigel containing VEGF (100 ng) and 10 or 20 μM of xanthorrhizol and curcumin respectively. After 7 days, mice were killed and Matrigel plugs were excised. Representative Matrigel plugs that contained no VEGF (control), VEGF alone, or VEGF plus xanthorrhizol and curcumin were photographed and weighed (A). Quantification of neovessel formation by measurement of hemoglobin in the Matrigel (B). After injection of Matrigel alone or containing VEGF, the mice were treated daily with xanthorrhizol and curcumin at a dose of 0.02 mg/kg by oral gavage. The control mice received only PBS. At day 7, plugs were collected, and the angiogenic response was evaluated by measuring the hemoglobin content of the plugs (C). The data are presented as mean±SE; #, p <0.01, ##, p <0.05 versus VEGF-non treated group and *,p <0.01, **, p <0.05 versus VEGF alone; bars

IV. DISCUSSION

Angiogenesis, the formation of new blood vessels from pre-existing endothelium, is a fundamental step in a variety of physiological and pathological conditions including wound healing, embryonic development, chronic inflammation, tumor progression, and metastasis. Solid tumors cannot grow beyond a certain size without inducing the formation of new blood vessels.[41] Angiogenesis, therefore, is essential for tumor growth and the pharmacological down-regulation of angiogenesis or the suppression of new vascular growth, is an important avenue for the development of a novel class of anticancer agents. Recently, considerable attention has been focused on the naturally occurring phytochemicals that exist in medicinal or edible plants as good sources of novel chemopreventive agents. It has been anticipated that phytochemicals with antioxidative and anti-inflammatory activity will exert chemopreventive effects in carcinogenesis, particularly in the promotion stage.

We have previously demonstrated that xanthorrhizol inhibited the COX-2 expression and NF-κB activation induced by TPA in mouse skin as well as the two-stage mouse skin carcinogenesis induced by DMBA-TPA. It has also been reported that xanthorrhizol has the potential to attenuate cisplatin-induced nephrotoxicity[42], and has an anti-metastatic potential in an experimental mouse lung metastasis model.[43]

Curcumin (diferuloylmethane) is a principal yellow pigment present in the rhizome of turmeric (*Curcuma longa* L). Previous studies have demonstrated anti-carcinogenic properties of curcumin in animals, including the inhibition of tumor initiation induced by benzo-(a)pyrene and tumor promotion induced by phorbol esters on mouse skin.[44, 45]

In the present study, we provide direct evidence, for the first time, that xanthorrhizol has potent antiangiogenic activity *in vitro* and *in vivo*. Our results support the tumor-preventive action of xanthorrhizol. We also investigated the

antiangiogenic effect of curcumin in order to compare it with xanthorrhizol. Xanthorrhizol is derived from the same genus as curcumin which has already been developed as a chemopreventive agent.

VEGF is generated from a variety of tumors and is the most important angiogenic factor associated with induction and maintenance of the neovasculature in human tumors.[46] Thus, the effects of xanthorrhizol and curcumin on VEGF-induced angiogenesis were investigated in this study.

At the cellular level, xanthorrhizol and curcumin almost completely suppressed the stimulatory effect of VEGF on endothelial cell proliferation, migration, and tube formation, which are the key events in the angiogenesis process. Xanthorrhizol and curcumin inhibited VEGF-induced HUVECs proliferation in a dose-dependent manner, with half maximal inhibition at 10 μM and 5 μM, respectively (Figure 2A), and also inhibited VEGF-induced DNA synthesis of HUVECs (Figure 2B). Endothelial cell migration, which is another key step in angiogenesis, was also inhibited by xanthorrhizol and curcumin in a dose-dependent manner (Figure 3). Consistent with these results, tube formation, one of the characteristic features of endothelial cells during angiogenic development, was blocked by both xanthorrhizol and curcumin. At a dose of 20 μM, xanthorrhizol and curcumin completely eliminated the tube formation of endothelial cells (Figure 4).

The serine/threonine protein kinase, Akt, is a downstream effector of PI3k that is activated by a variety of growth factors including those known to induce angiogenesis, such as VEGF and bFGF.[47] Constitutive active PI3k and Akt induce angiogenesis, and the inhibition of PI3k signaling interferes with angiogenesis. PI3k signaling also mediates VEGF expression in endothelial cells, suggesting possible involvement of the PI3k-Akt pathway in the angiogenic process.[48] In addition to its survival-promoting effect, Akt has recently been shown to promote endothelial cell migration by phosphorylating and activating eNOS, which leads to an increase in NO production and upregulation of the intracellular adhesion molecule-1 (ICAM-1)

expression in rat primary brain microvascular endothelial cells (BMEC).[49] In this study, we found that VEGF increased Akt (Ser473) and eNOS (Ser1177) phosphorylation strongly at 30 min, and that xanthorrhizol and curcumin significantly diminished VEGF-induced Akt and eNOS phosphorylation at the same time point in a dose-dependent manner without inducing a change in the total Akt and eNOS protein levels. There were slight decreases in total Akt and eNOS when treated with 20 μM of curcumin. Wortmannin also down-regulated VEGF-induced Akt and eNOS phosphorylation (Figure 5, Figure 6). These results suggest that xanthorrhizol and curcumin may exert their antiangiogenic ability by interfering with the PI3k/Akt/eNOS signaling pathway.

There are evidences that adhesion molecules may play a role in angiogenesis. A study by Radisavljevic *et al.*[49] showed that vascular endothelial growth factor-mediated endothelial cell migration involves increased ICAM-1 expression through an Akt/NO-dependent pathway. Nguyen M *et al.*[50] showed that antibodies directed against E-selectin inhibited the formation of capillary-like tubes *in vitro*. Therefore, we investigated whether xanthorrhizol and curcumin inhibited adhesion molecules (ICAM-1, VCAM-1, and E-selectin) in VEGF-stimulated HUVECs. We found that VEGF enhanced the expression of all three adhesion molecules. Maximal expressions of the adhesion molecules were reached by 6 h (Figure 7). Interestingly, xanthorrhizol inhibited VCAM-1 and E-selectin expressions in a dose-dependent manner but showed relatively weaker activity in the case of ICAM-1, whereas curcumin inhibited expression of three molecules tested (Figure 8). Additionally, we also used PI3k inhibitor, wortmannin, nitric oxide synthase (NOS) inhibitor, L-NAME, NF-κB inhibitor, parthenolide, and PKC inhibitor, chelerythrine chloride to investigate the major mechanism leading the induction of adhesion molecules on VEGF-induced HUVECs (Figre 9). Unexpectedly, wortmannin enhanced VEGF-stimulated expression of ICAM-1, VCAM-1, and E-selectin. Parthenolide, however, completely suppressed the VEGF-induced expression of VCAM-1 and

E-selectin comparable to ICAM-1, whereas L-NAME did not produce any effect on the expression of those adhesion molecules. The induction of NO may not be involved in the regulation of these adhesion molecules. It has been reported that VEGF-stimulated expression of ICAM-1, VCAM-1, and E-selectin mRNA was mainly regulated through NF-κB activation with PI3k suppression, but was independent of nitric oxide and MEK. They showed that insulin, PI3k activator, suppressed both basal and VEGF-stimulation of adhesion molecules expression.[51] Consistent with above report, our results suggested that the VEGF-stimulated expression of ICAM-1, VCAM-1, and E-selectin proteins may be mainly mediated through the activation of NF-κB, along with PI3k-mediated suppression.

Ludwig *et al.*[52] demonstrated that epigallocatechin-3-gallate (EGCG), the major component in green tea, selectively reduced cytokine-induced VCAM-1, but not ICAM-1 and the E-selectin surface expression in HUVECs. Babita *et al.*[53] reported that diferuloylmethane is the most active component present in the ethyl acetate extract of *Curcuma longa* L. for inhibiting TNF-α induced ICAM-1, VCAM-1, and E-selectin expression in HUVECs at doses of 40 μM. Toyoaki *et al.*[54] demonstrated that inhibition of endogenous NO synthesis by L-NAME inhibited endothelial expression of integrin $\alpha_V\beta_3$ in both normoxic and hypoxic conditions. In contrast, L-NAME did not alter expression of other potentially angiogenesis-related cell adhesion molecules such as PECAM-1, VCAM-1, ICAM-1, connexin 43, or VE-cadherin.

The NF-κB family of transcription factors is known to participate in the regulation of numerous genes that are required for cell growth and survival. NF-κB is a heterodimer, mostly composed of p65 and p50 subunits and retained in the cytoplasm as an inactive form through interaction with IκB inhibitory proteins, such as IκBα.[55] Because previous results showed that the expression of adhesion molecules is mainly regulated by NF-κB, we examined whether xanthorrhizol and curcumin inhibited nuclear translocation of NF-κB subunits (Figure 10). Xanthorrhizol and curcumin decreased nuclear levels of both VEGF-induced p50 and p65 in

VEGF-stimulated HUVECs. The decrease in protein levels of both subunits occurred at a lower dose of xanthorrhizol, which was more effective than that of curcumin at the same dose. To further prove the transcriptional level of NF-κB by xanthorrhizol and curcumin treatment, transient transfection and reporter gene assay were performed by using a promoter/reporter plasmid construct containing the human NF-κB gene fused to a reporter, luciferase. Significant decreases in NF-κB promoter transcriptional activity by xanthorrhizol and curcumin in a presence of VEGF were observed (Figure 10C). In contrast to previous adhesion molecule expression data, wortmannin decreased VEGF-induced p65 and p50 subunits, suggesting a different target or targets for its action other than PI3k, which is upstream as well as directly/indirectly linked to the NF-κB pathway. These results suggest the need for further investigation of this phenomenon. In recent report, preincubation of HUVECs with 100 μM of EGCG for 1 h prior to TNF-α stimulation did not significantly prevent the nuclear translocation of p65 and p50.[52] In our results, however, xanthorrhizol completely decreased protein levels of both subunit at the dose of 10 μM. It has been well accepted that NF-κB signaling pathway plays important role in the cancer biology. So, we expect that xanthorrhizol will exert its anticancer activity in diverse field including control of cell growth, apoptosis, inflammation, stress response and many other physiological processe.

It is important to know about the anti-angiogenic effect with phytochemicals administration, because good candidate for anti-angiogenic drugs include those available for oral administration for a long term without severe systemic side effects. Thus, we investigated the effects of xanthorrhizol and curcumin on angiogenic differentiation using an *in vivo* Matrigel plug model and CAM assay. This is the first time an *in vivo* assay model has shown the anti-angiogenic activity of xanthorrhizol and that this can be achieved by oral dosing. Both xanthorrhizol and curcumin significantly suppressed the induction of new blood formation in the Matrigel plug implanted in C57BL/6 mice in response to VEGF (Figure 12), and inhibited the

formation of blood vessels in the CAM assay (**Figure** 11). I**n the CAM assay,** x**anthorrhizol seemed to be more effective than the positive control, retinoic acid** (**Figure** 11). **These antiangiogenic activities of** x**anthorrhizol and curcumin *in vivo* may be e**x**plained by their inhibitory action on proliferation, migration, and differentiation of endothelial cells in response to angiogenic growth factors such as** V**EGF**.

Ⅴ. CONCLUSION

In conclusion, the pleiotropic antiangiogenic effects of xanthorrhizol and curcumin involve the inhibition of growth and capillary tube organization and reduced the migration of human endothelial cells. The molecular events associated with these effects include the downregulation of the phosphorylation of Akt and eNOS; inhibition of ICAM-1 and VCAM-1, and E-selectin expression; and inhibition of NF-κB signaling. Additionally, we also observed the antiangiogenic activities of xanthorrhizol and curcumin in mouse Matrigel plugs and CAM assays. Taken together, these findings provide a mechanistic rationale for the development of xanthorrhizol and curcumin as orally administered angiopreventive/antiangiogenic agents for clinical use.

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ABSTRACT (IN KOREAN)

Xanthorrhizol과 **curcumin**의 신생혈관 억제 능 및 세포내 신호전달 체계를 통한 작용기전

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김 미 정

신생혈관형성(**angiogenesis**)은 기존의 미세혈관으로부터 새로운 모세혈관이 생성되는 과정으로서 종양의 성장과 전이에 중요한 단계이다. 신체내의 정상적인 생리조건에서 혈관내피세포의 증식은 엄격히 조절되는데 반해 당뇨병성 망막증, 자궁내막증, 건선, 동맥경화증, 암과 같은 병리학적 조건에서는 무절제한 혈관신생이 일어난다. 특히 고형 암의 성장에 필수적으로 신생혈관이 발생한다. 이에 혈관신생을 억제하는 물질 개발이 암 치료에 있어 중요한 부분으로 인식되고 있다.

Vascular Endothelial Growth Factor (**VEGF**)는 혈관신생 촉진 인자로 널리 알려져 있다. 특히 혈관내피세포의 유사분열촉진인자와 화학 주성인자의 기능을 가지고 있으며 상처 치유, 신생혈관, 허혈성 조직, 종양 성장, 혈관 보호, 지혈과정에 관련되어 있고, 혈관내피세포의 생존을 촉진한다.

Phosphatidylinositol 3-kinase (**PI3k**)는 **cell adhesion, vesicular trafficking, protein synthesis** 그리고 **cell survival**과 같은 많은 세포내 기능에 관련되어 있다. **PI3k**의 **downstream target**인 **serine-threonine kinase**인 **Akt**는 **phosphoinositide-dependent protein kinase** (**PDK**)에 의해 **Thr-308, Ser-473**에 **phosphorylation**됨에 따라 활성화 되어 **cell survival, glycogen metabolism,**

cellular transformation 그리고 **myogenic differentiation**을 조절한다.

Nitric Oxide (NO)는 혈관내피세포의 생존에 중요한 역할을 하고 있다. 이는 **Nitric Oxide Synthase (NOS)**에 의해 생성되는데, 특히 혈관 내에서 생성되는 **eNOS**는 **VEGF**에 의해 **Akt**에 의존적으로 **Ser-1177**에 인산화가 유도되어 신생혈관에 관여한다고 알려져 있다. 또한 세포 표면에 존재하는 **adhesion molecule** 역시 세포의 이동과 관계되어 혈관신생에 영향을 미친다. **Adhesion molecule**의 종류에는 **intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VAM-1), E-selection** 등이 존재한다.

Xanthorrhizol은 ***Curcuma xanthorrhiza*** **Roxb,**의 뿌리줄기에서 추출한 **sesquiterpenoid**로서 인도네시아에서 전통적으로 식용, 의약용으로 사용되어 왔다. ***Curcuma longa*** **L,** 의 뿌리줄기에서 분말화 한 삼황 분말의 활성 물질인 **curcumin**은 커리의 독특한 색과 향에 주요 성분 이다. **Curcumin** 역시 염증을 치료하기 위해 민간요법으로 사용되어 왔고, 항산화 작용, 항염증 작용이 보고 된 바 있다.

본 연구에서는 **xanthorrhizol**과 **curcumin**의 신생혈관 억제 능을 ***in vitro, in vivo*** 상에서 확인하였다. 혈관형성의 주 세포가 되는 혈관내피세포를 이용하여 ***in vitro*** 상에서 혈관형성 과정에서 필수적인 단계가 되는 혈관내피세포의 증식, 혈관 고리 형성, 이동이 억제되는 것을 확인하였고, 계란의 융모요막을 이용하여 혈관신생억제를 확인할 수 있는 **CAM assay**와 **Matrigel**을 이용한 **mice Matrigel plug assay**를 이용하여 ***in vivo*** 상에서 **xanthorrhizol**과 **curcumin**의 신생혈관억제효능을 확인하였다. 또한 세포 생존과 관련되어 있어 혈관신생을 유도하는 것으로 알려져 있는 **PI3k**와 이의 **downstream effector**인 **Akt**와 세포의 생존, 그리고 특히 세포의 이동에 관련을 하고 있는 **eNOS**를 **protein** 수준에서 억제되는 것을 확인하였으며, **adhesion molecule**의 **protein** 발현을 **NF-κB**에 의존적으로 억제시키는 것을 확인하였다. 위와 같은 결과로, 식물 유래화합물인 **xanthorrhizol** 과 **curcumin**의 암을 치료하는데 있어 신생혈관억제제로서의 유용성을 제시하고 있다.

핵심되는 말 : 잔토리졸, 컬큐민, 혈관내피세포, 신생혈관, **PI3k/Akt/eNOS, adhesion molecules, NF-κB**