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Sanguiin H-6 Blocks Endothelial Cell Growth through Inhibition of VEGF Binding to VEGF Receptor

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The vascular endothelial growth factor (VEGF) plays a key role in angiogenesis, which is a process where new blood vessels develop from the endothelium of a pre-existing vasculature. VEGF exerts its activity by binding to its receptor tyrosine kinase, KDR/Flk-1, which is expressed on the surface of endothelial cells. A methanol extract and organic solvent (*n*-hexane, ethyl acetate, *n*-butanol, aqueous) fractions from *Rubus coreanus* were examined for their inhibitory effects on VEGF binding to the VEGF receptor. The methanol extract from the crude drug were found to significantly inhibit VEGF binding to the VEGF receptor ($IC_{50} \approx 27$ μg/mL). Among the fractions examined, the aqueous fraction from the medicinal plant showed potent inhibitory effects against the binding of KDR/Flk-1-Fc to immobilized $VEGF_{165}$ in a dose-dependent manner ($IC_{50} \approx 11$ μg/mL). Sanguiin H-6 was isolated as an active principle from the aqueous fraction, and inhibited the binding of KDR/Flk-1-Fc to immobilized $VEGF_{165}$ in a dose-dependent manner ($IC_{50} \approx 0.3$ μg/mL). In addition, sanguiin H-6 efficiently blocked the VEGF-induced HUVEC proliferation in a dose-dependent manner ($IC_{50} \approx 7.4$ μg/mL) but had no effect on the growth of HT1080 human fibrosarcoma cells. This suggests that sanguiin H-6 might be a potential anti-angiogenic agent.

Key words: VEGF, KDR/Flk-1, Angiogenesis, Cancer

INTRODUCTION

The growth of solid tumors depends on angiogenesis, which is the generation of new blood vessels from pre-existing vessels (Folkman, 1991). Tumors promote angiogenesis by secreting various growth factors that stimulate endothelial migration, proliferation, proteolytic activity, and capillary morphogenesis (Risau, 1990). Newly formed blood vessels supply a tumor with nutrients and oxygen, dispose of its metabolic waste products and generate paracrine stimuli, which further promote tumor cell proliferation and its invasiveness (Folkman, 1991; Nicosia *et al.*, 1983). Therefore, the inhibition of angiogenesis is an attractive approach for treating human cancers (Boehm-Viswanathan, 2000).

Among the known proangiogenic factors, the vascular endothelial growth factor (VEGF) is a potent endothelial cell-specific mitogen *in vitro*, and enhances the vascular permeability and stimulates angiogenesis *in vivo* (Gospodarowicz *et al.*, 1989; Leung *et al.*, 1989). VEGF exerts its activity by binding to its receptors, Flt-1 and KDR/Flk-1, which are expressed on the surface of endothelial cells (Millauer *et al.*, 1993). Gene deletion studies have demonstrated the significance of VEGF in vasculogenesis and angiogenesis (Shalaby *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). In addition, several molecules, which block the interaction between VEGF and KDR/Flk-1, including endostatin (Kim *et al.*, 2002), arginine-rich peptides (Bae *et al.*, 2000), (-)-epigallocatechin-3-gallate (Lamy *et al.*, 2002; Kondo, T. *et al.*, 2002), and 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose (Lee *et al.*, 2004) have been identified. Therefore, the VEGF/VEGF receptor system is an attractive target for inhibiting tumor angiogenesis and tumor growth (Veikkola and Alitalo, 1999; Ferrara, 2002).

Rubus coreanus was selected as part of an ongoing study aimed at identifying novels compounds from Chinese herb medicines with anti-angiogenic properties that block the interaction between VEGF and its receptor, KDR/Flk-1. Bioassay-guided fractionation, which led to the isolation of sanguiin H-6, was performed. The ellagitannin

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also inhibits the VEGF-induced proliferation of human endothelial cells.

MATERIALS AND METHODS

Materials

The unripe fruits of *Rubus coreanus* along with other medicinal plants were purchased from various herbal markets in Seoul, Korea. The specimens were authenticated by Dr. S-Y, Hwang, Korea Medical Science Institute Co. Ltd. The fetal bovine serum (FBS), M199 and RPMI 1640 were purchased from Invitrogen (Grand Island, NY). The recombinant human VEGF and basic fibroblast growth factor (bFGF) were obtained from R&D Systems (Minneapolis, MN). The chemiluminescence ELISA substrate and sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) were acquired from Roche (Mannheim, Germany). All the other reagents were purchased from Sigma (St. Louis, MO).

Plant extracts

The medicinal plant was extracted with methanol at room temperature. The methanol extracts were dried under reduced pressure, and partitioned into n-hexane, ethyl acetate, *n*-butanol, and water fractions. The resulting fractions were then lyophilized and resuspended in dimethyl sulfoxide (DMSO).

Purification of Sanguiin H-6

The unripe fruits of *Rubus coreanus* (3 kg) were extracted three times with methanol and partitioned into *n*-hexane, ethyl acetate, *n*-butanol, and water fractions. Sanguiin H-6 (83 mg) was purified using a reverse phase C18 column with water/MeOH mixtures (100 : 0 → 0 : 100) and water/acetonitrile mixtures (100 : 0 → 0 : 100) from the aqueous fraction. The structure of Sanguiin H-6 (Fig. 1) was identified using ^{1}H- nuclear magnetic resonance (NMR), ^{13}C-NMR, and electron ionization mass spectroscopy together with the published data (Nonaka *et al.*, 1982).

Sanguiin H-6

IR ν_{max} (KBr): 3430 (OH), 1748 (C=O), 1610, 1518, 1447 (aromatic C=C), 1045 (glycosidic OH); ^{1}H-NMR (400 MHz, DMSO-d_6) : 3.81 (1H, d, *J*=13 Hz, H-6'), 3.91 (1H, d, *J*=13 Hz, H-6), 4.22 (1H, m, H-5), 4.36 (1H, m, H-5'), 5.03 (1H, t, *J*=9 Hz, H-4), 5.11 (2H, t, *J*=9 Hz, H-3, H-4'), 5.20 (1H, t, *J*=9 Hz, H-2'), 5.24 (1H, dd, *J*=7, 13 Hz,, H-6'), 5.29 (1H, dd, *J*=4, 9 Hz, H-2), 5.37 (1H, t, *J*=9 Hz, H-3'), 5.57 (1H, dd, *J*=6, 13 Hz, H-6), 6.17 (1H, *J*=9 Hz, H-1'), 6.31, 6.39, 6.47, 6.51 [each 1H, s, hexahydroxydiphenoyl (HHDP)-H], 6.54 (1H, d, *J*=4 Hz, H-1), 6.77, 6.78 (each 1H, s, HHDP-H and sanguisoboyl-H), 7.11 (2H, s, galloyl-H), 7.13, 7.27 (each 1H, d, *J*=2 Hz, sanguisorboyl-H).

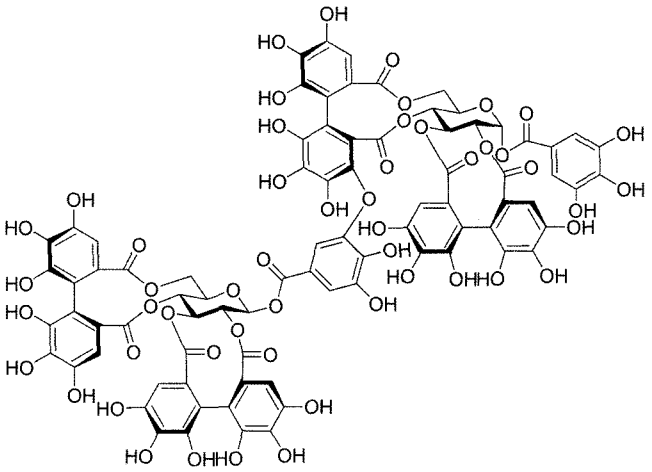

Fig. 1. The chemical structure of sanguiin H-6.

Binding of KDR/Flk-1-Fc to immobilized VEGF

A $VEGF_{165}$ (80 ng/well) solution in 100 μL of PBS was immobilized in 96-well plates. The wells were washed and blocked with 3% BSA in PBS for 2 h. The mixture (100 μL) was added to each well after preincubating the KDR/Flk-1-Fc (30 ng/mL) in 0.3% BSA/PBS with or without various amountc of PGG, EGCG, gallic acid, and gallacetophenone for 10 min. All experiments were carried out in the presence of an appropriate amount of DMSO. After 2 h, the wells were washed three times with PBST [PBS + 0.05% Tween 20]. The bound KDR/Flk-1-Fc was determined by incubation with the anti-human IgG-HRP followed by incubation with a chemiluminescent substrate. All experiments were carried out at room temperature. Each data point was assayed in triplicate.

Cell culture

The primary human umbilical vein endothelial cells (HUVECs) were prepared as described elsewhere (Kim *et al.*, 2002a), and maintained on gelatin-coated dishes in M199 supplemented with 20% FBS, 5 units/mL of heparin, 5 ng/mL of bFGF, and penicillin/streptomycin. The immortalized human microvascular endothelial cells (HMEC-1s) were maintained as described previously (Kim *et al.*, 2002a). The HT1080 human fibrosarcoma and DU-145 human prostate carcinoma cells were maintained in RPMI 1640 medium supplemented with 10% FBS and penicillin-streptomycin.

Cell proliferation assays

The HUVECs were seeded onto gelatin-coated 24-well plates at a density of 2.0×10^4 cells/well. After 24 h, the medium was replaced with M199 containing 5% FBS and

10 ng/mL of $VEGF_{165}$ with or without various amounts of PGG. After 72 h, the cells were trypsinized, and the total number of cells were counted. For the XTT assay, the HT1080 cells were seeded onto 96-well plates at a density of 5.0×10^3 cells/well. After 24 h, the medium was replaced with RPMI 1640 containing 5% FBS with or without various amounts of Sanguiin H-6. After 72 h, an XTT incorporation assay was carried out according to the manufacture's instructions. All the experiments were carried out in the presence of an appropriate amount of DMSO. Each data point was assayed in triplicate.

Statistical analysis

All values are expressed as a mean ± SD. The *P* values were calculated from the Student's *t* test, based on comparisons with the appropriate control samples examined at the same time. A P value < 0.05 was considered significant.

RESULTS AND DISCUSSION

The effect of the MeOH extract from the crude drug was first examined in order to identify molecules that block the interaction between VEGF and its receptor, KDR/Flk-1, from *Rubus coreanus* fruits. As shown Fig. 2, this extract inhibited the binding of KDR/Flk-1-Fc to the immobilized $VEGF_{165}$ in a dose-dependent manner ($IC_{50} \approx 27$ μg/mL). This extract was further fractionated into *n*-hexane, EtOAc, *n*-BuOH, and aqueous fractions for activity-guided fractionation. The aqueous fraction showed most potent inhibitory activity of the four fractions (Fig. 3, $IC_{50} \approx 11$ μg/mL). The other fractions showed weak inhibitory activity (< 40% at 100 μg/mL). Further fractionation of the aqueous fraction led to the isolation of sanguiin H-6 (Fig. 1), which significantly inhibited the interaction between VEGF and KDR/Flk-1-Fc. As shown in Fig. 4, sanguiin H-6 inhibited the binding of KDR/Flk-1-Fc to immobilized $VEGF_{165}$ in a dose-dependent manner ($IC_{50} \approx 0.3$ μg/mL).

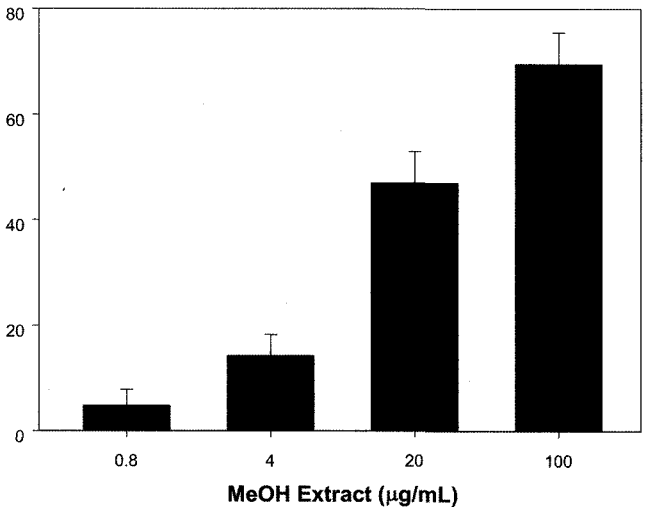

Fig. 2. Effect of the MeOH extract from *Rubus coreanus* on the binding of KDR/Flk-1-Fc to the immobilized VEGF. KDR/Flk-1-Fc was added with various concentration of the extract to the $VEGF_{165}$ coated 96-well plates. After incubation, the amount of bound KDR/Flk-1-Fc was determined. The MeOH extract from the crude drug inhibited the binding of KDR/Flk-1-Fc in a dose-dependent manner.

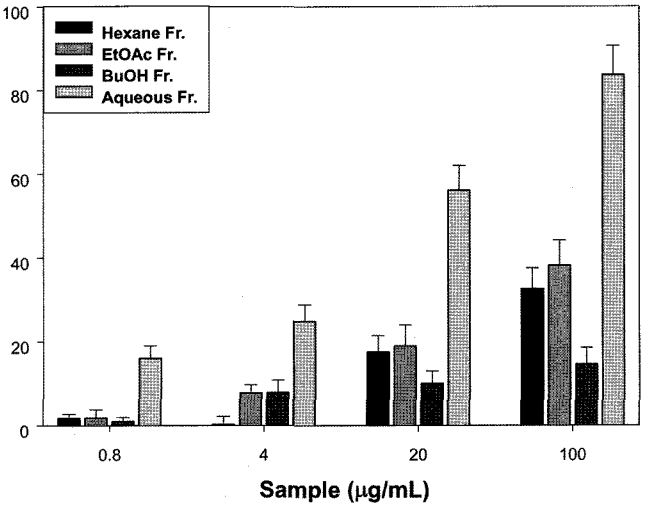

Fig. 3. Effects of the four solvent fractions from *Rubus coreanus* on the binding of KDR/Flk-1-Fc to immobilized VEGF. The aqueous fraction from the crude drug inhibited the binding of KDR/Flk-1-Fc in a dose-dependent manner.

This study investigated the effects of sanguiin H-6 on the VEGF-induced endothelial cell proliferation Because VEGF induces the proliferation, migration and differentiation of endothelial cells via the activation of KDR/Flk-1 (Millauer *et al.*, 1993). As shown in Fig. 5, sanguiin H-6

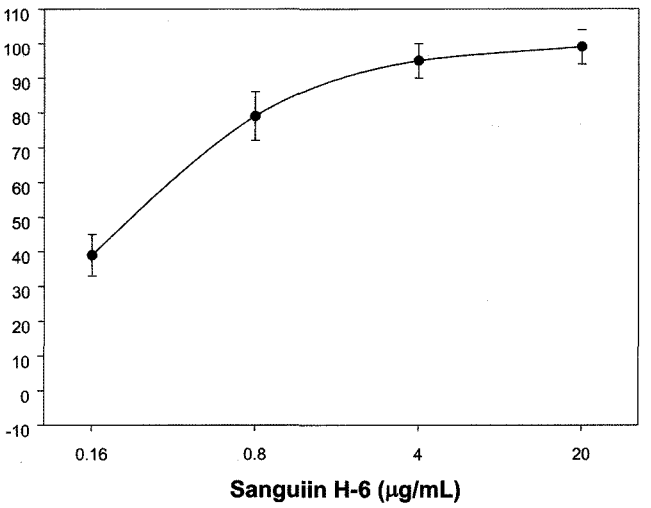

Fig. 4. Effect of sanguiin H-6 on the binding of KDR/Flk-1-Fc to the immobilized VEGF. Sanguiin H-6 inhibited the binding of KDR/Flk-1-Fc in a dose-dependent manner.

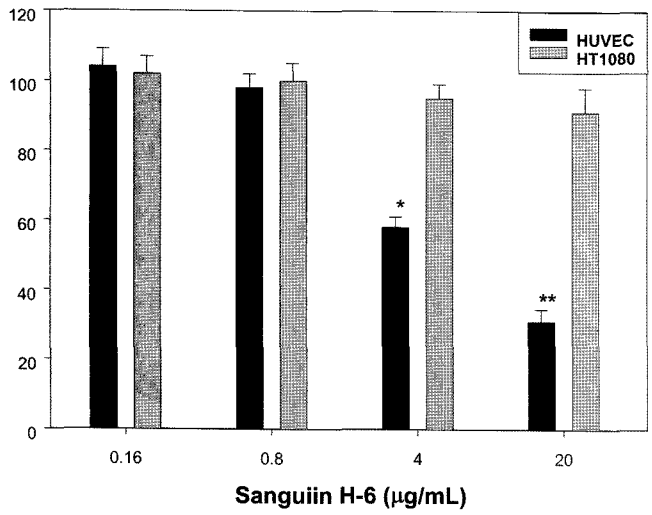

Fig. 5. Effects of sanguiin H-6 on cell growth. Sanguiin H-6 blocked theVEGF-induced endothelial cell proliferation. HUVECs were seeded onto 24-well plates at 2.0×10^4 cells and incubated with M199 containing 5% FBS and 10 ng/mL of VEGF in the presence or absence of sanguiin H-6. After incubation for 72 h, the total number of cells was counted using a microscope. The HT1080 cells were treated with sanguiin H-6 at the indicated concentrations. After incubation for 72 h, the cell density was assessed using XTT assay. *, $P < 0.05$; **, $P < 0.01$ vs. sanguiin H-6-untreated group.

efficiently blocked the VEGF-induced HUVEC proliferation in a dose dependent manner ($IC_{50} \approx 7.4$ μg/mL). The effect of sanguiin H-6 on the proliferation of cancer cells was also investigated. Sanguiin H-6 at concentrations up to 20 μg/mL did not affect the growth of HT1080 human fibrosarcoma cells (Fig. 5). This suggests that sanguiin H-6 has stronger anti-proliferative activity in endothelial cells than in cancer cells.

It was reported that sanguiin H-6 not only inhibits the expression of inducible nitric oxide synthase (iNOS) mRNA in a dose-dependent manner but also inhibits iNOS activity (Yokozawa *et al.*, 2002). Angiogenesis that is mediated *via* VEGF-independent mechanisms appears to involve nitric oxide (NO). Monocyte-induced angiogenesis is L-arginine/NO dependent (Leibovich *et al.*, 1994). NO also mediates *in vivo* angiogenesis and *in vitro* endothelial cell proliferation and migration promoted by substance P (Ziche *et al.*, 1994). These studies strongly suggest that the proper modulation of NO is vital for angiogenesis. Therefore, sanguiin H-6 may contribute to the inhibition of endothelial cell proliferation by reducing the level of NO production by inhibiting iNOS activity and mRNA expression.

It was reported that tea catechins including EGCG inhibit KDR/Flk-1 phosphorylation and block angiogenesis *in vitro via* the inhibition of VEGF receptor binding (Kondo *et al.*, 2002; Lamy *et al.*, 2002,) EGCG, gallic acid, and 1,2,3,4,6-penta-O-galloyl-beta-D-glucose (PGG), in which the glucose core is saturated with galloyl groups, inhibit the interaction between VEGF and KDR/Flk-1 (Lee *et al.*, 2004). Sanguiin H-6 has a molecular weight of 1,870, and is consists of four hexahydroxyphenoyl, two galloyl, and two glucosyl units. This indicates that the galloyl unit may be essential for the anti-tumor activity of gallotannins and ellagitannins.

It was reported that oxidant stress enhances angiogenesis (Khatri *et al.*, 2004). Sanguiin H-6 was reported to be a major contributor to the antioxidant capacity of *Rubus idaeus* L. (Mullen *et al.*, 2002). Therefore, the antioxidant activity of sanguiin H-6 might contribute to its anti-angiogenic effect.

In conclusion, sanguiin H-6 from the unripe fruits of *Rubus coreanus* Miq. (Rosaceae) blocked the binding of $VEGF_{165}$ to KDR/Flk-1 and reduced the VEGF and bFGF-induced endothelial cell proliferation. ELISA binding assay of VEGF binding to KDR/Flk-1 may be a useful screening method for the development of anti-VEGFR binding agents against many angiogenesis-dependent diseases.

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REFERENCES

Bae, D. G., Gho, Y. S., Yoon, W. H., and Chae, C. B., Arginine-rich anti-vascular endothelial growth factor peptides inhibit tumor growth and metastasis by blocking angiogenesis. *J. Biol. Chem.*, 275, 13588-13596 (2000).

Boehm-Viswanathan, T., Is angiogenesis inhibition the Holy Grail of cancer therapy? *Curr. Opin. Oncol.*, 12, 89-94 (2000).

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., and Nagy, A., Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*, 380, 435-439 (1996).

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J., and Moore, M. W., Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*, 380, 439-442 (1996).

Ferrara, N., VEGF and the quest for tumor angiogenesis factors. *Nat. Rev. Cancer*, 2, 795-803 (2002).

Folkman, J., What is the evidence that tumors are angiogenesis-dependent? *J. Natl. Cancer Inst.*, 82, 4-6 (1991).

Gospodarowicz, D., Abraham, J. A., and Schilling, J., Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. *Proc.*

Natl. Acad. Sci. U.S.A., 86, 7311-7315 (1989).

Khatri, J.J., Johnson, C., Magid, R., Lessner, S. M., Laude, K. M., Dikalov, S. I., Harrison D.G., Sung H. J., Rong, Y., and Galis Z. S., Vascular oxidant stress enhances progression and angiogenesis of experimental atheroma. *Circulation*, 109, 520-525 (2004).

Kim, C. W., Lee, H. M., Lee, T. H., Kang, C., Kleinman, H. K., and Gho, Y. S., Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. *Cancer Res.*, 62, 6312-6317 (2002a).

Kim, Y. M., Hwang, S., Kim, Y. M., Pyun, B. J., Kim, T. Y., Lee, S. T., Gho, Y. S., and Kwon, Y. G., Endostatin blocks vascular endothelial growth factor-mediated signaling *via* direct interaction with KDR/Flk-1. *J. Biol. Chem.*, 277, 27872-27879 (2002b).

Kondo, T., Ohta, T., Igura, K., Hara, Y., and Kaji, K., Tea catechins inhibit angiogenesis *in vitro*, measured by human endothelial cell growth, migration and tube formation, through inhibition of VEGF receptor binding. *Cancer Lett.*, 180, 139-144 (2002).

Lamy, S., Gingras, D., and Beliveau, R., Green tea catechins inhibit vascular endothelial growth factor receptor phosphorylation. *Cancer Res.*, 62, 381-385 (2002).

Lee, S. J., Lee, H. M., Ji, S. T., Lee, S. R., Mar, W., and Gho, Y. S., 1,2,3,4,6-Penta-*O*-galloyl-beta-D-glucose blocks endothelial cell growth and tube formation through inhibition of VEGF binding to VEGF receptor. *Cancer Lett.*, 208, 89-94 (2004).

Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Frrarara, N., Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*, 246, 1306-1309 (1989).

Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N. P., Risau, W., and Ullrich, A., High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell*, 72, 835-846 (1993).

Ncosia, R. F., Tchao, R., and Leighton, J., Angiogenesis-dependent tumor spread in reinforced fibrin clot culture. *Cancer Res.*, 43, 2159-2166 (1983).

Nonaka, G. I., Tanaka, T., Nita, M., and Nishioka, I., A dimeric hydrolysable tannin, sanguiin H-6 from *Sanguisorba officinalis* L. *Chem. Pharm. Bull.*, 30, 2255-2257 (1982).

Risau, W., Angiogenic growth factors. *Prog. Growth Factor Res.*, 2, 71-79 (1990).

Shalaby, F., Rossant, J., Yamaguch, T. P., Gertsenstein, M., Wu, X.F., Breitman, M. L., and Schuh, S. C., Failure of blood-island formation vasculogenesis in Flk-1-deficient mice. *Nature*, 376 62-66 (1995).

Veikkola, T. and Alitalo, K., VEGFs, receptors and angiogenesis. *Semin. Cancer Biol.*, 9, 211-220 (1999).