Natural Product Sciences
13(2) : 128-131 (2007)

Inhibitory Effects of *Paeonia suffruticosa* Andrews Extracts on VEGF Binding to VEGF Receptor

Sung-Jin Lee*, Hak-Kyo Lee

Gyeonggi Regional Research Center, Hankyong National University, Ansung, Gyeonggi 456-749, Korea

Abstract – Tumor angiogenesis is a critical step for the growth and metastasis of solid tumors. Vascular endothelial growth factor (VEGF) is the most important angiogenic molecule associated with tumor-induced neovascularization. VEGF exerts its activity through binding to its receptor tyrosine kinase, KDR/Flk-1, expressed on the surface of endothelial cells. This study was carried out to investigate inhibitory effect of extracts from root cortex of *Paeonia suffruticosa* Andrews on VEGF binding to VEGF receptor. The MeOH extract from *P. suffruticosa* Andr. inhibited the binding of KDR/Flk-1-Fc to immobilized $VEGF_{165}$ more than 45% at the concentration of 100 μg/mL. The MeOH extract was further fractionated into *n*-hexane, ethyl acetate, *n*-BuOH, and aqueous fractions. Among the four fractions, the ethyl acetate fraction from the root cortex of *P. suffruticosa* Andr. exhibited highly effective inhibition (≈ 79% inhibition) and then *n*-BuOH fraction (≈ 45% inhibition) on the binding of KDR/Flk-1-Fc to immobilized $VEGF_{165}$ at the concentration of 100 μg/mL. The ethyl acetate fraction from the root cortex of *P. suffruticosa* Andr. more efficiently blocked VEGF-induced human umbilical vein endothelial cell proliferation, than the growth of HT1080 human fibrosarcoma. Our results suggest that *P. suffruticosa* Andr. may be used as a candidate for developing anti-angiogenic agent.
Keywords – *Paeonia suffruticosa* Andrews, VEGF, angiogenesis

Introduction

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

Among a large number of proangiogenic factors, vascular endothelial growth factor (VEGF) is a potent endothelial cell-specific mitogen *in vitro* and stimulates angiogenesis *in vivo* (Leung *et al.*, 1989). VEGF exerts its activity through binding to its receptors, Flt-1 and KDR/Flk-1 expressed on the surface of endothelial cells (De Vries *et al.*, 1992; Millauer *et al.*, 1993). Gene deletion studies of VEGF and its receptors have demonstrated the significance of VEGF/VEGF receptor system in vasculogenesis and angiogenesis (Shalaby *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Neutralizing anti-VEGF monoclonal antibodies, and the expression of sFlt-1 and dominant-negative KDR/Flk-1 on cells in the vicinity of growing tumors block tumor growth and metastasis (Kim *et al.*, 1993; Millauer *et al.*, 1994; Goldman *et al.*, 1998). Furthermore, several molecules including endostatin, arginine-rich peptides and (−)-epigallocatechin-3-gallate (EGCG) that block the interaction of VEGF with KDR/Flk-1, VEGF-induced angiogenesis and tumor growth have been identified (Kim *et al.*, 2002b; Bae *et al.*, 2000; Lamy *et al.*, 2002; Kondo *et al.*, 2002). Thus, the VEGF/VEGF receptor system is an attractive target for inhibition of tumor angiogenesis, tumor growth, and metastasis.

Moutan Cortex Radicis, the root cortex of *Paeonia suffruticosa* Andrews (Ranunculaceae), is an important herb medicine used in Chinese traditional medicine as both an analgesic and an anti-inflammatory agent (Lin *et al.*, 1998), and it is prescribed in various Chinese preparations for the treatment of blood stagnation. It has been reported that the MeOH extract of Moutan Cortex Radicis increases antioxidant activity (Lee *et al.*, 2003),

*Author for correspondence
Fax: +82-31-670-5491; E-mail: genielee@hknu.ac.kr

prevents the process of herpes simplex virus attachment and penetration (Hsiang *et al.*, 2001) and inhibits enzymes crucial to the life cycle of the human immunodeficiency virus (HIV) (Au *et al.*, 2001).

In the present study, we investigated the inhibitory activity of solvent fractions from the root cortex of *P. suffruticosa* Andr. on VEGF binding to VEGF receptor.

Experimental

Materials – The root cortex of *Paeonia suffruticosa* Andrews was purchased from herb markets in Seoul, Korea. The medicinal plant was extracted with MeOH at room temperature. The MeOH extract was dried under reduced pressure, and then the concentrated MeOH extract was partitioned into *n*-hexane, ethyl acetate, *n*-BuOH, and aqueous fractions. The fractions were lyophilized, resuspended in dimethyl sulfoxide (DMSO). Fetal bovine serum (FBS), M199 and RPMI 1640 were purchased from Invitrogen (Grand Island, NY). Recombinant human VEGF was from R&D Systems (Minneapolis, MN). Chemiluminescence ELISA substrate and sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) were purchased from Roche (Mannheim, Germany). All the other reagents were purchased from Sigma (St. Louis, MO).

Binding of KDR/Flk-1-Fc to immobilized VEGF – $VEGF_{165}$ (80 ng/well) in 100 μL of PBS were immobilized to 96-well plates. The wells were washed and blocked with 3% BSA in PBS for 2 h. After 10 min preincubation of KDR/Flk-1-Fc (30 ng/mL) in 0.3% BSA/PBS with or without various amount of sample, the mixture (100 μL) was added to each well. All experiments were carried out in the presence of 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 anti-human IgG-HRP and followed by a chemiluminescent substrate. All experiments were carried out at room temperature. Each data point was assayed in triplicate.

Cell culture – Primary human umbilical vein endothelial cells (HUVECs) were prepared as described previously (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. HT1080 human fibrosarcoma cells were maintained in RPMI 1640 medium supplemented with 10% FBS and penicillin-streptomycin.

Cell proliferation assays – HUVECs were seeded at a density of 2.0×10^4 cells/well onto gelatin-coated 24-well plates. After 24 h, the medium was replaced with M199 containing 5% FBS and 10 ng/mL of $VEGF_{165}$ with or without various amount of sample. After 72 h, the cells were trypsinized and the total number of cells was counted. For XTT assay, cancer cells were seeded at a density of 5.0×10^3 cells/well onto 96-well plates. After 24 h, the medium was replaced with RPMI 1640 containing 5% FBS with or without various amount of sample. After 72 h, XTT incorporation assay was carried out according to the manufacture's instruction. All experiments were carried out in the presence of appropriate amount of DMSO. Each data point was assayed in triplicate.

Statistical analysis – All values are expressed as mean ± SD. *P* values were calculated from the Student's *t* test, based on comparisons with appropriate control samples tested at the same time.

Results and discussion

To search the molecules that block the interaction between VEGF and its receptor KDR/Flk-1 from the root cortex of *Paeonia suffruticosa* Andrews, we first examined the effect MeOH extract from the crude drug. As shown Fig. 1, this extract inhibited the binding of KDR/Flk-1-Fc to immobilized $VEGF_{165}$ more than 45% at the concentration of 100 μg/mL and its inhibitory activity was dose-dependent. The MeOH extract was further fractionated into *n*-hexane, ethyl acetate, *n*-BuOH, and aqueous fractions.

We next investigated the effects of the four fractions from the root cortex of *P. suffruticosa* Andr. on the binding of KDR/Flk-1-Fc to immobilized $VEGF_{165}$. As shown in Fig. 2, the ethyl acetate fraction exhibited highly effective inhibition (≈ 79% inhibition) and then *n*- BuOH fraction showed 45% inhibition rate at the concentration of 100 μg/mL. Its inhibitory activity was dose-dependent. However *n*-hexane and aqueous fractions showed weak inhibitory activities (less than 15% at the concentration 100 μg/mL).

Because VEGF induces proliferation, migration and differentiation of endothelial cells through activation of KDR/Flk-1 (Millauer *et al.*, 1993), we investigated the effects of the ethyl acetate fraction from the root cortex of *P. suffruticosa* Andr. on the VEGF-induced endothelial cell proliferation. As shown in Fig. 3, treatment of the ethyl acetate fraction showed a significant inhibition on the VEGF-induced HUVEC proliferation (≈ 45% inhibition at the concentration of 20 μg/mL, $p < 0.05$). Its inhibitory activity was dose-dependent. We also investigated the effect of the ethyl acetate fraction on proliferation of cancer cells. The ethyl acetate fraction showed ≈ 28%

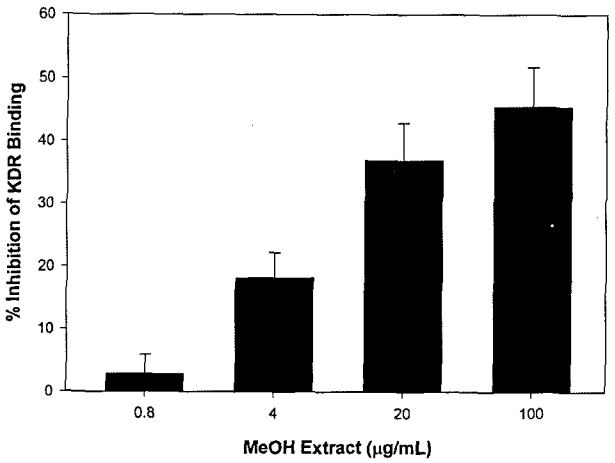

Fig. 1. Effect of MeOH extract from the root cortex of *Paeonia suffruticosa* Andrews on the binding of KDR/Flk-1-Fc to immobilized VEGF. KDR/Flk-1-Fc with various concentration of the extract was added to the $VEGF_{165}$ coated 96-well plates. After incubation, the bound KDR/Flk-1-Fc was determined. MeOH extract from *P. suffruticosa* Andr. inhibited the binding of KDR/Flk-1-Fc in a dose-dependent manner.

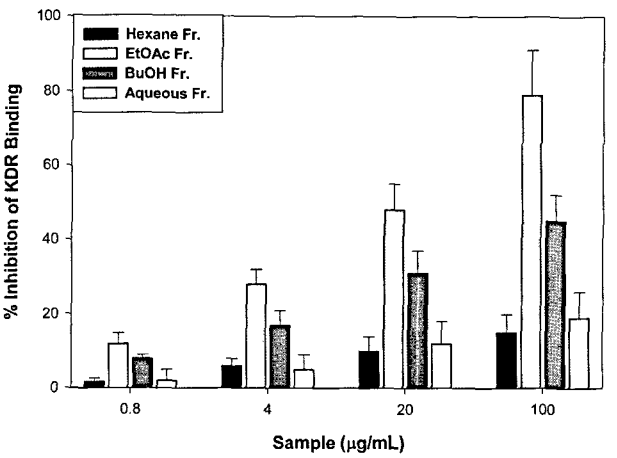

Fig. 2. Effects of four solvent fractions from the root cortex of *Paeonia suffruticosa* Andrews on the binding of KDR/Flk-1-Fc to immobilized VEGF. The ethyl acetate (EtOAc) fraction inhibited the binding of KDR/Flk-1-Fc in a dose-dependent manner.

inhibition on HT1080 human fibrosarcoma cell proliferation at the concentration of 20 μg/mL. These results suggest that the ethyl acetate fraction showed more strong anti-proliferative activity on endothelial cells than on cancer cells

It has been known that oxidant stress enhances angiogenesis (Khatri *et al.*, 2004). Lee *et al.* (2003) reported that the MeOH extract of Moutan Cortex Radicis shows antioxidant activity. The antioxidant activity of sanguiin H-6 may contribute to its anti-angiogenic effect.

It has been reported that paeonol from the root cortex of *P. suffruticosa* Andr. not only inhibit the expression of

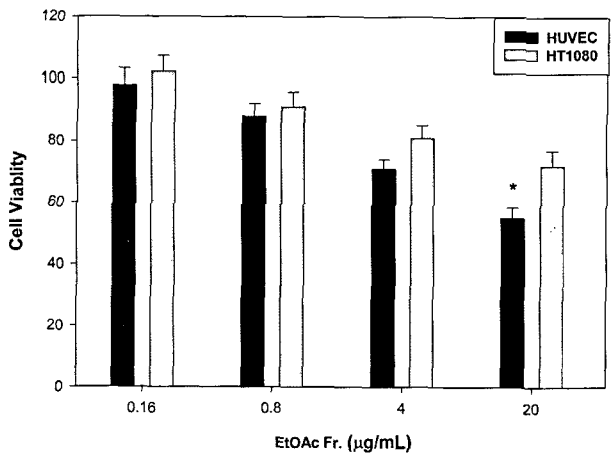

Fig. 3. Effects of the ethyl acetate (EtOAc) fraction from the root cortex of *Paeonia suffruticosa* Andrews on cell growth. The ethyl acetate fraction blocked VEGF-induced endothelial cell proliferation. HUVECs seeded at 2.0×10^4 cells onto 24-well plates were incubated with M199 containing 5% FBS and 10 ng/mL of VEGF in the presence or absence of the fraction. After incubation for 72 h, total cell number was counted under a microscope. HT1080 cells were treated with the ethyl acetate fraction at concentrations as indicated. After incubation for 72 h, cell density was assessed by XTT assay. *, $P < 0.05$ vs. the ethyl acetate fraction from the root cortex of *P. suffruticosa* Andr. untreated group.

inducible nitric oxide synthase (iNOS) mRNA in a dose-dependent manner, but also inhibit iNOS activity (Park and Choi, 2005). Angiogenesis mediated via VEGF-independent mechanisms appears to involve nitric oxide (NO). Monocyte-induced angiogenesis is L-arginine/NO dependent (Leibovich *et al.*, 1994). *In vivo* angiogenesis and *in vitro* endothelial cell proliferation and migration promoted by substance P are also mediated by NO (Ziche *et al.*, 1994). These studies suggest strongly that proper modulation of NO is vital for angiogenesis. Therefore, the root cortex of *P. suffruticosa* Andr. may contribute to the inhibition of endothelial cell proliferation by reducing NO production through inhibition of iNOS activity and mRNA expression.

Previous studies indicated that several inhibitors of α-glucosidase block angiogenesis. α-Glucosidase inhibitor castanospermine alters endothelial cell glycosylation, reduces ability to migrate and to invade basement membrane gels *in vitro* and inhibits tumor growth (Pili *et al.*, 1995). α-Glucosidase inhibitor 1,6-epi-cyclophellitol inhibits metastasis (Atsumi *et al.*, 1993). It has been reported that the ethyl acetate fraction from the root cortex of *P. suffruticosa* Andr. was shown to have an inhibitory effect on α-glucosidase *in vitro* as well as on blood glucose elevation in mice loaded with maltose *in vivo* (Lee and Ji, 2005). Therefore α-glucosidase inhibitor may be useful for the screening of angiogenesis inhibitor.

In this report, we present that the effects of the ethyl acetate fraction from the root cortex of *Paeonia suffruticosa* Andrews (Ranunculaceae) block the binding of $VEGF_{165}$ to KDR/Flk-1 and reduce VEGF-induced endothelial cell proliferation. These results suggest that *P. suffruticosa* Andr. may be used as a candidate for developing anti-angiogenic agent. However, the mechanism of action and the nature of the active molecules remain to be elucidated. So, further analysis of the herb medicine extracts, including additional purification and chemical characterization, should permit the identification of these interesting principles possessing inhibitory activity on the binding of $VEGF_{165}$ to KDR/Flk-1 and VEGF-induced endothelial cell proliferation.

Acknowledgements

The research was supported by the "GRRC" Project of Gyeonggi Provincial Government, Republic of Korea.

References

Atsumi, S., Nosaka, C., Ochi, Y., Iinuma, H., and Umezawa, K., Inhibition of experimental metastasis by an α-glucosidase inhibitor, 1,6-epi-cyclophellitol. *Cancer Res.* **53**, 4896-4899 (1993).

Au, T.K., Lam, T.L., Ng, T.B., Fong, W.P., and Wan, D.C., A Comparison of HIV-1 integrase inhibition by aqueous and methanol extracts of Chinese medicinal herbs. *Life Science* **68**, 1687-1694 (2001)

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).

De Vries, C.J., Escobedo, A., Ueno, H., Houck, K., Ferrara, N., and Williams, L.T., The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor, *Science* **255**, 989-991 (1992).

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).

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

Goldman, C.K., Kendall, R.I., Cabrera, G., Soroceanu, L., Heike, Y., Gillespie, G.Y., Siegel, G.P., Mao, X., Bett, A.J., Huckle, W.R., Thomas, K.A., and Curiel, D.T., Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate, *Proc. Natl. Acad. Sci. USA* **95**, 8795-8800 (1998).

Hsiang, C.Y., Hsieh, C.L., Wu, S.L., Lai, I.L., and Ho, T.Y., Inhibitory effect of anti-pyretic and anti-inflammatory herbs on herpes simplex virus replication. *Am. J. Chin. Med.* **29**, 459-467 (2001).

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, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., and Ferrara, N., Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo, *Nature* **362**, 841-844 (1993).

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.E., Hwang, H.J., Ha, J.S., Jeong, H.S., and Kim, J.H., Screening of medicinal plant extracts for antioxidant activity. *Life Sci.* **73**, 167-79 (2003).

Lee, S.J. and Ji, S.T., Inhibitory Effects of *Paeonia suffruticosa* extracts on maltase and Sucrase. *Nat. Prod. Sci.* **11**, 127-130 (2005)

Leibovich, S.J., Polverini, P.J., Fong, T.W., Harlow, L.A., and Koch, A.E., Production of angiogenic activity by human monocytes requires an L-arginine/nitric oxide-synthase-dependent effector mechanism. *Proc. Natl. Acad. Sci. USA* **91**, 4190-4194 (1994).

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).

Lin, H.C., Ding, H.Y., and Wu, Y.C., Two novel compounds from *Paeonia suffructicosa. J. of Nat. Prod.* **61**, 343-346 (1998).

Millauer, B., Shawver, L.K., Plate, K.H., Risau, W., and Ullrich, A., Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant, *Nature* **367**, 576-579 (1994).

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).

Park, H.J. and Choi, M.Y., In vitro antiinflamatory activity of paeonol from the essential oil and its derivative methylpaeonol. *Kor. J. Pharmacogn.* **26**, 116-120 (2005).

Pili, R., Cang, J., Partis, R.A., Mueller, R.A., Chrest, F.J., and Passaniti, A. The α-glucosidase inhibitor castanospermine alters endothelial cell glycosylation, prevents angiogenesis, and inhibits tumor growth. *Cancer Res.* **55**, 2920-2926 (1995).

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).

Ziche, M., Morbidelli, L., Masini, E., Amerini, S., Granger, H.J., Maggi, C.A., Geppetti, P., and Ledda, F., Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *J. Clin. Invest.* **94**, 2036-2044 (1994).

(Accepted April 13, 2007)