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Inhibitory Effects of *Rehmannia glutinosa* Liboschitz on Endothelial Cell Proliferation

Sung Jin Lee† **and Hak Kyo Lee**

Gyeong-gi Regional Research Center, Hankyong National University, Anseong, Gyeonggi 456-749, Korea.

ABSTRACT : Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are the most important angiogenic molecules associated with tumor-induced neovascularization. This study was carried out to investigate inhibitory effect of extracts from root of *Rehmannia glutinosa* LIBOSCHITZ (*Rehmannia Radix* and *Rehmannia Radix* Preparata) on endothelial cell proliferation. The methanol extracts from the medicinal herb were fractionated into *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions. Among the four fractions, the *n*-butanol fraction from *R. Radix* on exhibited highly effective inhibition (≈79% inhibition) on the binding of KDR/Flk-1-Fc to immobilized $VEGF_{165}$ and then ethyl acetate fraction from *R. Radix* (≈45% inhibition) at the concentration of 100 μg/mℓ. The *n*-butanol fraction efficiently blocked the VEGF- and bFGF-induced HUVEC proliferation in a dose-dependent manner, but did not affect the growth of HT1080 human fibrosarcoma cells. The *n*-butanol fraction more efficiently blocked the binding of KDR/Flk-1-Fc to immobilized $VEGF_{165}$ and VEGF- and bFGF-induced human umbilical vein endothelial cell proliferation than the fraction from *R. Radix* Preparata. Our results suggest that *Rehmannia Radix* may be used as a candidate for developing anti-angiogenic agent.

Key words : *Rehmannia glutinosa*, VEGF, angiogenesis

INTRODUCTION

Growth of solid tumors depends on angiogenesis, the generation of new blood vessels from pre-existing vessels (Folkman, 1991). When the balance between angiogenic and angiostatic factors is disrupted, tumor cells may begin to release uncontrolled angiogenic factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). These factors go on to stimulate endothelial cell proliferation, and newly formed endothelial cells break down the extracellular matrix, migrate to cancer cells (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, 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).

The fibroblast growth factors (FGFs) are a distinct class of potent mitogens (Folkman & Klagsbrun 1987, Thomas 1987). These factors bind to heparin sulfate proteoglycans (Vlodavsky *et al.* 1987) and stimulate endothelial cells to migrate and form tubes (Montesano *et al.* 1986).

Rehmannia glutinosa LIBOSCHITZ, is an important medicinal herb used in Chinese traditional medicine. It has been reported that the polysaccharides isolated from the root of *R. glutinosa* LIBO. show the properties of immunomodulation (Tomoda et al., 1994; Kim et al., 1999) and antitumor (Wei and Ru, 1997) and antioxidant (Cho, 2003).

In the present study, we investigated the inhibitory activity of solvent fractions from the root of *R. glutinosa* LIBO. on endothelial cell proliferation.

MATERIALS AND METHODS

Materials

The root of *Rehmannia glutinosa* LIBOSCHITZ was purchased from herb markets in Seoul, Korea. The medicinal plant was extracted with methanol at room temperature. The methanol extracts were dried under reduced pressure, and then the concentrated methanol extracts were partitioned into n-hexane, ethyl acetate, *n*-butanol, and aqueous fractions. The fractions were lyophilized, resuspended in dimethyl sulfoxide (DMSO). Fetal bovine serum (FBS), M199 and RPMI 1640

† Corresponding author: (Phone) +82-31-670-5491 (E-mail) genielee@hknu.ac.kr
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were purchased from Invitrogen (Grand Island, NY). Recombinant human VEGF and basic fibroblast growth factor (bFGF) were from R&D Systems (Minneapolis, MN). Chemiluminescence ELISA substrate and sodium 3'-[1-(pheny lamino-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 $\mu\ell$ 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 the fraction from the root of *R. glutinosa* LIBO. 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., 2002) and maintained on gelatin-coated dishes in M199 supplemented with 20% FBS, 5 units/mℓ of heparin, 10 ng/mℓ of VEGF or 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 $VEGF_{165}$ or 5 ng/mℓ of bFGF with or without various amount of the solvent fraction from the root of *R. glutinosa* LIBO. For XTT assay, HT1080 human fibrosarcoma 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 the solvent fraction from the root of *R. glutinosa* LIBO. 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

Because previous studies have reported that VEGF induces proliferation and migration through activation of its cell surface receptor, KDR/Flk-1 (Veikkola and Alitalo, 1999), we investigated the effects of *Rehmannia glutinosa* LIBOSCHITZ (*Rehmannia Radix* and *Rehmannia Radix* Preparata) on the binding of $VEGF_{165}$ to KDR/Flk-1 in enzyme-linked immunosorbent assay (ELISA) binding assay.

To screen the medicinal herb extracts that block the interaction between VEGF and its receptor KDR/Flk-1 and endothelial cell proliferation, we first examined the effect of methanol extracts from t from *Rehmannia Radix* and *Rehmannia Radix* Preparata on the binding of KDR/Flk-1-Fc to immobilized VEGF165. The methanol extracts from *Rehmannia Radix* (≈ 42% inhibition) and *Rehmannia Radix* Preparata (≈21% inhibition) showed inhibitory activity at the concentration of 100 μg/mℓ.

The methanol extracts were further fractionated into *n*-hexane, ethyl acetate, *n*-butanol, and aqueous fractions. We investigated the effects of the four fractions from the medicinal herb on the binding of KDR/Flk-1-Fc to immobilized $VEGF_{165}$. As shown in Fig. 1, the *n*-butanol fraction from *Rehmannia Radix* exhibited highly effective inhibition (≈79% inhibition) and then the ethyl acetate fraction (≈45% inhibition) from *R. Radix* at the concentration of 100 μg/mℓ. However *n*-hexane and aqueous fractions (less than 15%) from *R. Radix* and the four fractions (less than 40%) from *R. Radix* Preparata showed weak inhibitory activities at the concentration 100 μg/mℓ.

Because VEGF induces proliferation, migration and differ-

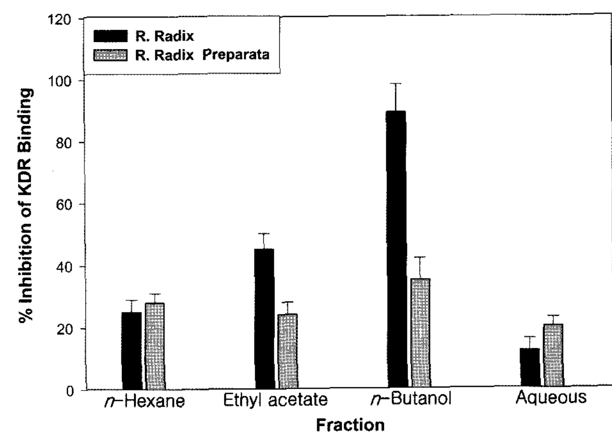

Fig. 1. Effect of four solvent fractions from the root of *Rehmannia glutinosa* LIBOSCHITZ (*Rehmannia Radix* and *Rehmannia Radix Preparata*) on the binding of KDR/Flk-1-Fc to immobilized VEGF at the concentration of 100 μg/mℓ.

entiation of endothelial cells through activation of KDR/Flk-1 (Millauer et al., 1993), we investigated the effects of the four fractions from *R. Radix* on the VEGF-induced endothelial cell proliferation. As shown in Fig. 2, treatment of the *n*-butanol fraction from *R. Radix* showed a significant inhibition on the VEGF-induced HUVEC proliferation (≈40% inhibition at the concentration of 100 μg/mℓ, $p < 0.05$).

Since basic fibroblast growth factor (bFGF) is a mitogen and a survival factor in endothelial cell (Folkman & Klagsbrun 1987, Thomas 1987), we investigated the effects of the four fractions from *R. Radix* on the bFGF-induced endothelial cell proliferation. As shown in Fig. 2, treatment of the *n*-butanol fraction from *R. Radix* showed a significant inhibition on the bFGF-induced HUVEC proliferation (≈31% inhibition at the concentration of 100 μg/mℓ, $p < 0.05$).

We also investigated the effect of the four fractions from the medicinal herb on proliferation of cancer cells. As shown in Fig. 2, the four fractions from *R. Radix* almost did not affect the growth of HT1080 human fibrosarcoma. Our results suggest that PGG showed more strong anti-proliferative activity on endothelial cells than on cancer cells.

In order to compare the results obtained the *n*-butanol fraction from *R. Radix* with those of *n*-butanol fraction from *R. Radix* Preparata, we investigated the effect of the *n*-butanol fraction from *R. Radix* and *R. Radix* Preparata on the VEGF- and bGFG-induced HUVEC proliferation. As shown in Fig. 3, *n*-butanol fraction from *R. Radix* showed more strong anti-proliferative activity on VEGF-induced endothelial cells than that of *n*-butanol fraction from *R. Radix* Preparata. Its inhibitory activity was dose-dependent. As shown in Fig. 4, *n*-butanol fraction from *R. Radix* also showed more strong anti-proliferative activity on bFGF-induced endothelial cells than that of *n*-butanol fraction from *R. Radix* Preparata.

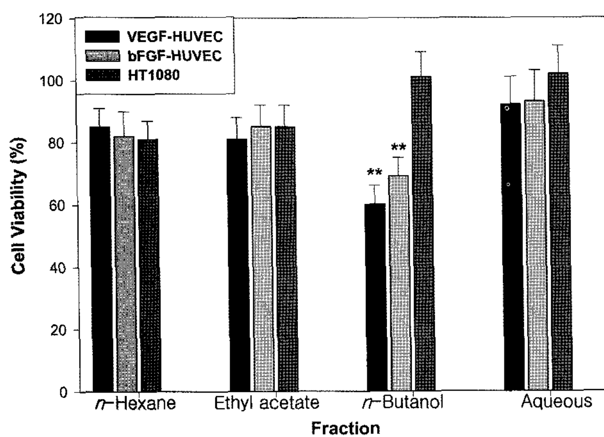

Fig. 2. Effects of four solvent fractions from *Rehmannia Radix* on VEGF- or bFGF-induced endothelial cell, or HT1080 human fibrosarcoma cell proliferation. **, $P < 0.01$ vs. HT1080 human fibrosarcoma cell viability.

It has been known that oxidant stress enhances angiogenesis (Khatri et al., 2004). Kim et al. (2005) reported that acteoside extracted from the leaves of *R. glutinosa* LIBO. increases antioxidant activity. The antioxidant activity of *R. glutinosa* LIBO. may contribute to its anti-angiogenic effect.

It has been reported that catalpol, an iridoid glycoside, contained richly in the roots of Rehmannia glutinosa significantly reduced the release of reactive oxygen species (ROS), TNF-alpha and NO after LPS-induced microglial activation and attenuated LPS-induced the expression of iNOS (Tian et al., 2006). Angiogenesis mediated via VEGF-independent mechanisms appears to involve nitric oxide (NO). Monocyte-induced

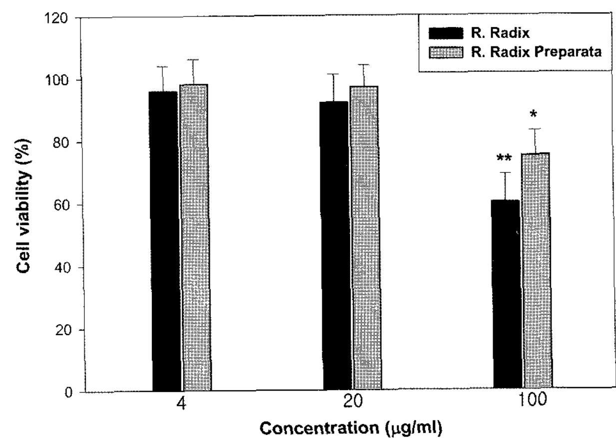

Fig. 3. Effects of the *n*-butanol fraction from the root of *Rehmannia glutinosa* LIBOSCHITZ (*Rehmannia Radix* and *Rehmannia Radix Preparata*) on VEGF-induced endothelial cell proliferation at concentrations as indicated. *, $P < 0.05$ vs. control group. **, $P < 0.01$ vs. control group.

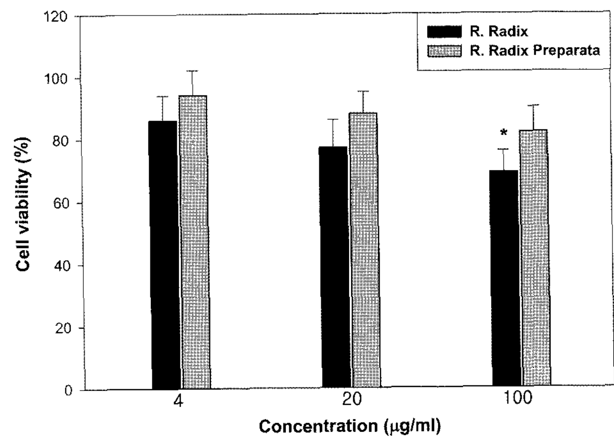

Fig. 4. Effects of the *n*-butanol fraction from the root of *Rehmannia glutinosa* LIBOSCHITZ (*Rehmannia Radix* and *Rehmannia Radix Preparata*) on bFGF-induced endothelial cell proliferation at concentrations as indicated. *, $P < 0.05$ vs. control group.

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, *R. Radix* may contribute to the inhibition of endothelial cell proliferation by reducing NO production through inhibition of iNOS activity and expression.

In this report, we present that the effects of the *n*-butanol fraction from the root of *R. glutinosa* LIBO. block the binding of $VEGF_{165}$ to KDR/Flk-1 and reduce VEGF-and bFGF-induced endothelial cell proliferation. These results suggest that the root of *R. glutinosa* LIBO. 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 medicinal herb 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.

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