Broussonetia kazinoki modulates the expression of VEGFR-2 and MMP-2 through the inhibition of ERK, Akt and p70^{S6K}-dependent signaling pathways: Its implication in endothelial cell proliferation, migration and tubular formation

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Abstract. *Broussonetia kazinoki* (BK) has been used as a traditional medicine to improve vision, as well as for inflammatory and infectious diseases. In the present study, we investigated the effects and molecular mechanism of the ethanolic extract of BK on cell proliferation, migration and tubular formation in vascular endothelial growth factor-A (VEGF-A)-treated human umbilical vein endothelial cells. BK treatment inhibited VEGF-A-stimulated endothelial cell proliferation through the downregulation of cell cycle-related proteins including cyclin-dependent kinases and cyclins. Moreover, BK treatment suppressed cell migration and tubular formation in response to VEGF-A. These anti-angiogenic activities of BK were associated with the inactivation of mitogenic signaling pathways including extracellular signal-regulated kinase, Akt and p70^{S6K}, and the subsequent downregulation of VEGFR-2 and matrix metalloproteinase-2. Taken together, these findings suggest further evaluation and development of BK as a potential therapeutic agent for the treatment and prevention of angiogenesis-related diseases including cancer.

Introduction

Angiogenesis, the formation and recruitment of new blood vessels, is known to play important roles in cancer growth and progression, and is regulated by the production of several angiogenic and anti-angiogenic factors (1,2). Among angiogenic factors, vascular endothelial growth factor-A (VEGF-A) and VEGF receptor-2 (VEGFR-2) signaling pathways stimulate the secretion and activation of matrix metalloproteinases (MMPs) in endothelial cells which result in cell proliferation, migration and survival, and are widely appreciated as the therapeutic targets for a variety of angiogenesis-related diseases. The central role of the VEGF-A/VEGFR-2 in this process is evidenced by the development and approval of pegaptanib and ranibizumab for age-related macular degeneration as well as bevacizumab and sunitinib for cancer treatment (3-5). However, current approved anti-angiogenic drugs frequently lead to drug resistance and recurrence of cancer growth and progression. Thus, further understanding of the molecular mechanisms and targets of angiogenic responses may help to develop potential therapeutic strategies for the treatment of cancer as well as angiogenesis-related diseases.

MMPs belong to a family of zinc-dependent endopeptidases and are responsible for tissue remodeling by selective proteolytic degradation, resulting in the promotion of cancer growth and progression through alterations of cell adhesion, migration, epithelial to mesenchymal transition, tumor angiogenesis and release of growth factors (6-8). These MMPs are regulated by their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (9,10). In addition to MMP-inhibitory activity, TIMPs have been reported to regulate cell growth, migration and differentiation through the MMP-independent mechanism (11-14). Identification of molecular mechanisms and targets in regulating expression and activities of MMPs and TIMPs within the tumor microenvironment has become an attractive strategy for therapeutic intervention in cancer growth and progression.

Broussonetia kazinoki (BK) Siebold (Moraceae) has been used as a traditional medicine for the amelioration of vision, inflammatory and infectious diseases as well as a material for production of paper in Northeastern Asia. Previous investigations have demonstrated that the extracts and bioactive phytochemicals of BK have anti-diabetic, anti-hyperglycemic,

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anti-inflammatory, anti-allergic and anticancer activities (15-19). However, the effects and molecular mechanisms of BK on angiogenesis remain unknown. In the present study, we evaluated the effects and signaling pathways of BK on proliferation, migration and tubular formation in human umbilical vein endothelial cells.

Materials and methods

Cell culture conditions. Primary cultures of human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA) and used between passages 4 and 6 for all the experiments. The cells were cultured in EGM-2® BulletKit media, according to the manufacturer's instructions (Lonza).

Reagents. The following pharmacological agents and antibodies were purchased from commercial sources: vascular endothelial growth factor-A (VEGF-A) and LY294002 (Merck Millipore, Billerica, MA, USA); rapamycin (Sigma-Aldrich, St. Louis, MO, USA); PD98059, anti-phospho-ERK (T202/Y204), anti-phospho-Akt (S473), anti-phospho-$p70^{S6K}$ (T421/S424), anti-phospho-$p38^{MAPK}$ (T180/Y182), anti-phospho-pRb (S780) and anti-MMP-2 (Cell Signaling Technology, Beverly, MA, USA); anti-VEGFR-2, anti-ERK, anti-Akt, anti-$p70^{S6K}$, anti-$p38^{MAPK}$, anti-TIMP-2, anti-Cdk4, anti-Cdk2, anti-cyclin D, anti-cyclin E, anti-actin antibodies and mouse and rabbit IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Preparation of Broussonetia kazinoki (BK) extract. The ethanol extract was prepared by mixing 100 g of twigs of BK with 1 liter of 100% ethanol and stirring for 72 h. The extract was then filtered through a filter paper (Advantec No. 1; Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and the filtrate was concentrated using a rotary evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 40˚C under vacuum. The yield of the dried extract was ~1.37%.

Cell viability and proliferation assay. HUVECs, plated on 6-well plates ($1x10^5$ cells/well; BD Biosciences, Bedford, MA, USA), were serum-starved for 14 h to synchronize cells in the G_1/G_0 phase of the cell cycle, and pretreated with BK (0.1-10 μg/ml) for 30 min in the presence or absence of PD98059 (25 μM), LY294002 (10 μM) or rapamycin (50 nM) as indicated, and then incubated with VEGF-A (10 ng/ml) for 24 h. Following culture for 24 h, cell viability was determined by a Muse™ cell analyzer using a cell count and viability assay kit (Merck Millipore), and the cell proliferation was quantified as previously described (20-22). The results from triplicate determinations (mean ± standard deviation) are presented as the number of cells per culture.

Western blot analysis. HUVECs in 100-mm dishes ($1x10^6$ cells/dish; BD Biosciences) were serum-starved for 14 h in endothelial cell basal medium (EBM-2; Lonza) and replaced with fresh media, followed by treatments for different time-points, as indicated in the figure legends, at 37˚C. The cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and lysed by incubation in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 100 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 80 mM β-glycerophosphate, 25 mM sodium fluoride and 1 mM sodium orthovanadate for 30 min at 4˚C. Cell lysates were clarified at 12,500 x g for 20 min at 4˚C, and the supernatants were subjected to western blot analysis as previously described (23,24). All the western blots were performed in triplicate and representative gels were shown.

Migration assay. Cell migration was quantified in the *in vitro* wound-healing assay as previously described (25,26). Cells were plated on 48-well plates ($4x10^4$ cells/well), grown to confluence, and a single wound was created in the center of the cell monolayer by the gentle removal of the attached cells with a sterile plastic pipette tip. Following serum starvation with EBM-2 for 2 h, the cells were pretreated with BK (0.1-10 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) stimulation for 16 h. The cells were fixed with methanol, and then stained with 0.04% Giemsa solution (Sigma-Aldrich). The migration of the cells into the wound was observed using images captured at the indicated time-points.

Tube formation assays. Matrigel® basement membrane matrix (10.4 mg/ml; BD Biosciences) was thawed overnight at 4˚C, and each well of pre-chilled 24-well plates was coated with 200 μl Matrigel® and then incubated at 37˚C for 30 min. Following serum starvation with EBM-2 for 2 h, the cells ($4x10^4$ cells/ml) were added to Matrigel®-coated plates and pretreated with BK (0.1-10 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) for 6 h. Tube formation was observed with an Olympus CKX41 inverted microscope (CAchN 10/0.25php objective) and ToupTek Toupview software (version x86, 3.5.563; Hangzhou ToupTek Photonics Co., Ltd., Zhejiang, China).

Zymogram analysis. Activities of MMPs were measured by zymography (27,28). Aliquots of conditioned medium collected from HUVECs treated with BK (10 μg/ml) and VEGF-A (10 ng/ml) for 16 h were diluted in sample buffer, and applied to 8% polyacrylamide gels containing 1 mg/ml gelatin (Sigma-Aldrich) as a substrate. After electrophoresis, the gels were incubated in 2.5% Triton X-100 for 1 h to remove SDS and allow the re-naturalization of MMPs, and then incubated in developing buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, and 150 mM NaCl for 16 h at 37˚C. The gels were stained with 0.5% Coomassie brilliant blue R-250 in 30% methanol-10% acetic acid for 3 h and followed by destaining with 30% methanol-10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of the Coomassie blue-stained gelatin.

Statistical analysis. Statistical analysis was performed using the Student's t-test, and was based on at least three different experiments. The results were considered to be statistically significant when $P<0.05$.

Results

BK suppresses VEGF-A-stimulated endothelial cell proliferation by modulating the expression of cell cycle-related

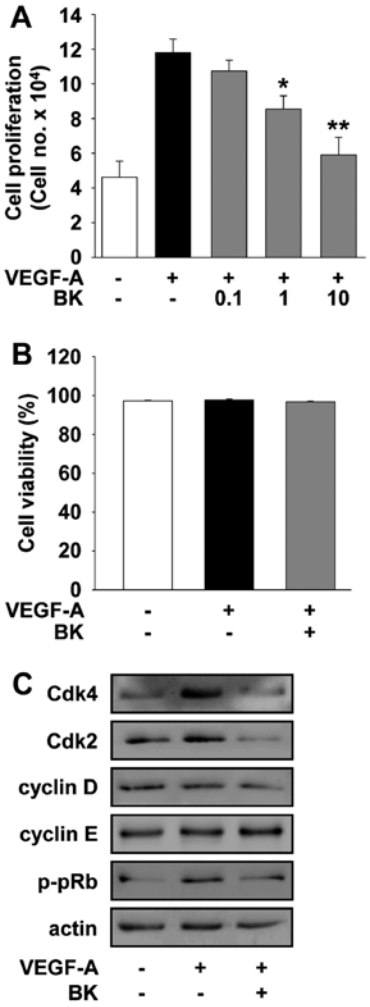

Figure 1. Anti-proliferative effect of BK on VEGF-A-stimulated HUVECs is mediated by downregulation of cell cycle-related proteins. (A) Quiescent cells were pretreated with or without BK at different concentrations (0.1-10 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) stimulation for 24 h. Values are the mean ± SD of three independent experiments. Statistical significance is indicated: *P<0.05, **P<0.01, compared with VEGF-A-treated cells. (B and C) Cells were pretreated with BK (10 μg/ml) for 30 min, followed by VEGF-A stimulation for 24 h. Cell viability and western blot analysis were performed as described in Materials and methods. (B) The results from triplicate determinations (mean ± SD) are presented as the percentage of viable cells of the total cell count. (C) Western blotting was performed on cell lysates with anti-Cdk4, anti-Cdk2, anti-cyclin D, anti-cyclin E, anti-phospho-pRb (S780) or anti-actin antibodies. Results shown are representative of at least three independent experiments.

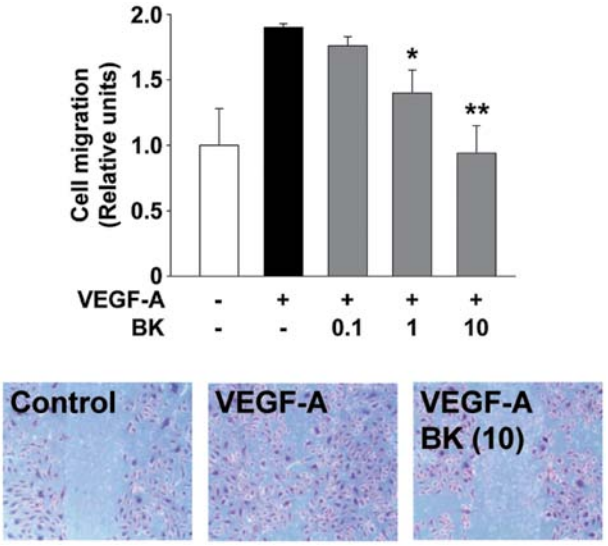

Figure 2. BK inhibits VEGF-A-stimulated migration in HUVECs. Cell migration was quantified by measuring the migration distance of cells from wound edge as described in Materials and methods. The cells were pretreated with BK (0.1-10 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) stimulation for 16 h. Results from six independent experiments (mean ± SD) were represented as the fold-increase of untreated controls. Statistical significance is indicated: *P<0.05, **P<0.01, compared with VEGF-A-treated cells.

proteins. We first examined the ability of BK to regulate proliferation in HUVECs. BK treatment suppressed VEGF-A-stimulated cell proliferation in a dose-dependent manner (Fig. 1A) and did not alter cell viability (Fig. 1B), suggesting that BK inhibition of cell proliferation is not mediated by the induction of apoptosis or cytotoxicity. Based on these findings, we examined the regulatory effect of BK on cell cycle progression by analyzing the changes of cyclin-dependent kinases (Cdks) and cyclins. Cell cycle progression requires activation of Cdks through formation with cyclins and subsequent phosphorylation of retinoblastoma protein (pRb) (29). As shown in Fig. 1C, BK treatment markedly suppressed the expression of Cdks and cyclin D, resulting in pRb hypophosphorylation in VEGF-A-treated HUVECs. These findings indicate that BK downregulates the expression of cell cycle-related proteins, resulting in inhibition of cell cycle progression and proliferation.

BK inhibits VEGF-A-stimulated endothelial cell migration and capillary structure formation. Endothelial cell migration and tubular formation are coordinately controlled by the interactions from ECM molecules to the intracellular and intercellular components of cells, and play important roles in angiogenic responses associated with cancer growth and progression (2). BK treatment dose-dependently inhibited VEGF-A-stimulated cell migration and tubular formation in HUVECs (Figs. 2 and 3). Collectively, these findings suggested that the bioactive components from the ethanolic extracts of BK act on multiple targets and mechanisms involved in cell proliferation, migration and tubular formation, resulting in the regulation of angiogenic responses *in vitro*.

BK suppresses VEGFR-2 and MMP-2 expression in VEGF-A-treated HUVECs. The expression and activation of VEGFR-2 and MMP-2 have been reported to promote angiogenic responses including endothelial cell proliferation, migration, invasion and tubular formation (1,2,30). To understand the molecular mechanism underlying the regulatory effects of BK on endothelial cell proliferation, migration and tubular formation, we analyzed the changes in the expression of VEGFR-2, MMP-2 and TIMP-2, an endogenous inhibitor of MMPs, in VEGF-A-treated HUVECs. BK treatment markedly suppressed the VEGF-A-induced expression of VEGFR-2 and MMP-2 (Fig. 4A). As shown in Fig. 4B, BK treatment also inhibited VEGF-A-induced activation of MMP-2 in conditioned medium of HUVECs. By contrast, the levels of TIMP-2 in HUVECs were not altered by VEGF-A or BK treatment (Fig. 4A). Taken

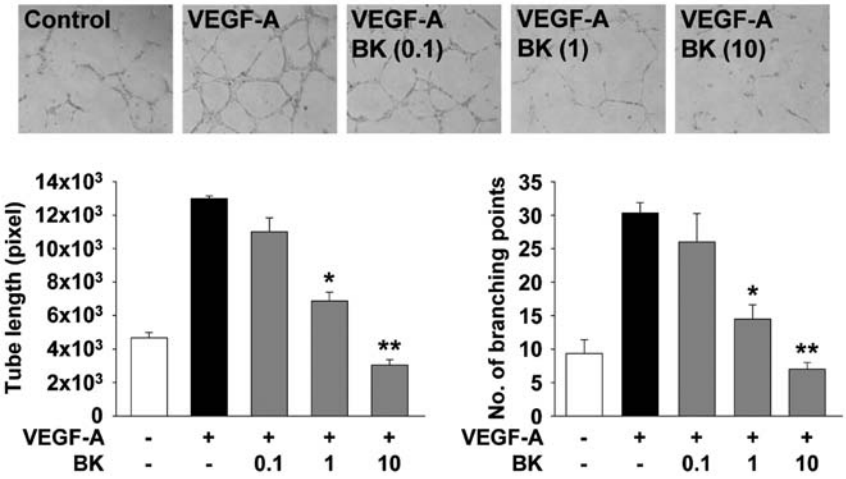

Figure 3. BK inhibits VEGF-A-stimulated formation of capillary structures in HUVECs. Tube formation was performed as described in Materials and methods, and quantified by measuring the lengths of tubes and number of branching points per unit area. The cells were pretreated with BK (0.1-10 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) stimulation for 6 h. Statistical significance is indicated: *P<0.05, **P<0.01, compared with VEGF-A-treated cells.

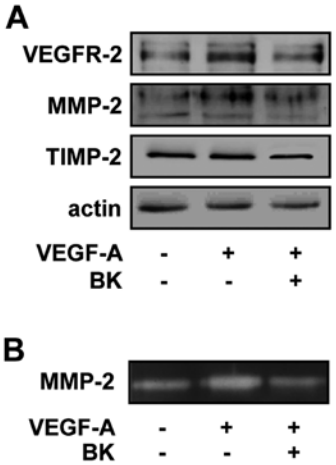

Figure 4. BK suppresses the VEGF-A-induced expression of VEGFR-2 and MMP-2. Quiescent cells were pretreated with BK (10 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) stimulation for (A) 24 h and (B) 16 h. (A) Western blotting was performed on cell lysates using anti-VEGFR-2, anti-MMP-2, anti-TIMP-2 or anti-actin antibodies. (B) Gelatin zymogram analysis was carried out by using conditioned medium from cell culture. Zymogram gel loading was normalized to total protein concentration. Results are representative of at least three independent experiments.

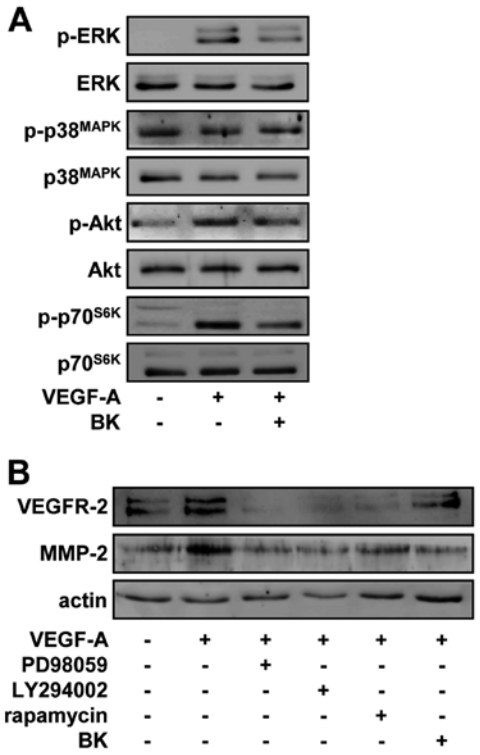

Figure 5. BK suppresses VEGFR-2 and MMP-2 expression through the inhibition of ERK, Akt and p70^{S6K} activity in VEGF-A-treated HUVECs. (A) Quiescent cells were pretreated with BK (10 μg/ml) for 30 min, followed by VEGF-A (10 ng/ml) stimulation for 15 min. Western blotting was performed on cell lysates using anti-phospho-ERK, anti-ERK, anti-phospho-p38^{MAPK}, anti-p38^{MAPK}, anti-phospho-Akt, anti-Akt, anti-phsopho-p70^{S6K} or anti-p70^{S6K} antibodies. (B) Cells were pretreated with PD98059 (25 μM), LY294002 (10 μM), rapamycin (50 nM) or BK (10 μg/ml) for 30 min, and then stimulated with VEGF-A (10 ng/ml) for 24 h. Western blotting was performed on cell lysates using anti-VEGFR-2, anti-MMP-2 or anti-actin antibodies. Results shown are representative of three independent experiments.

together, these findings indicates that the inhibitory effects of BK on endothelial cell proliferation, migration and tubular formation may be mediated at least in part through the suppression of VEGFR-2 and MMP-2 expression.

In vitro anti-angiogenic activities of BK are mediated through the inhibition of mitogenic signaling pathways and downregulation of VEGFR-2 and MMP-2. To investigate the molecular mechanisms by which BK regulates endothelial cell responses, we examined the changes in the activation of signaling pathways including extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38^{MAPK}), phosphatidylinositol 3-kinase (PI3-K)/Akt and mammalian target of rapamycin (mTOR)/p70^{S6K}, which play pivotal roles in cell fate (31). As shown in Fig. 5A, VEGF-A stimulation markedly increased the phosphorylation/activation of ERK, Akt and p70^{S6K}, but not that of p38^{MAPK}, when compared with unstimulated controls. By contrast, BK treatment significantly inhibited the VEGF-A-stimulated phosphorylation of ERK, Akt, and

$p70^{S6K}$ in HUVECs. Pretreatment of cells with PD98059 (an inhibitor of the ERK pathway), LY294002 (an inhibitor of the PI3-K/Akt pathway) or rapamycin (an inhibitor of the mTOR/$p70^{S6K}$ pathway) suppressed the expression of VEGFR-2 and MMP-2 in response to VEGF-A stimulation, suggesting that BK may contain pharmacologically effective components similar to these inhibitors, and share the roles and mechanisms of action in regulating endothelial cell proliferation, migration and tubular formation (Fig. 5B). Taken together, these findings demonstrated that the suppression of endothelial cell proliferation, migration and tubular formation by BK ethanol extracts might be mediated through the inactivation of VEGFR-2 downstream signaling pathways and subsequent downregulation of VEGFR-2 and MMP-2.

Discussion

Broussonetia kazinoki (BK) has been used as a traditional medicine for improving vision, as well as in inflammatory and infectious diseases. These applications are well supported by previous investigations that BK possesses bioactive phytochemicals such as isoprenylated and prenylated flavans to reduce inflammation (15,17). In addition to anti-inflammatory activity, BK has been reported to have cytotoxicity against several different cell lines including liver, cervical, oral, colon, lung and gastric cancer (18,19,32). However, no effects and molecular mechanisms of BK on angiogenesis have been reported thus far.

VEGF-A/VEGFR-2 signaling pathways play pivotal roles in the formation and stability of blood vessels associated with cancer growth and progression (1,3,14,30,33). These angiogenic responses induced by VEGF-A/VEGFR-2 signaling pathways include the secretion and activation of MMPs, resulting in the degradation of the extracellular matrix and remodeling of the tumor microenvironment. Thus, selective inhibition of VEGF-A/VEGFR-2 activation or downstream signaling pathways is appreciated as a potent strategy, compared to conventional chemotherapy.

To the best of our knowledge, in the present study, we have shown for the first time that the ethanol extract of BK inhibits VEGF-A-stimulated proliferation, migration and tubular formation in HUVECs. These anti-angiogenic activities of BK were found to be mediated through the inhibition of VEGF-A-stimulated phosphorylation/activation of ERK, Akt and $p70^{S6K}$, the downstream targets in VEGFR-2 signaling pathways, and downregulation of VEGFR-2 and MMP-2 as evidenced by using pharmacological inhibitors such as PD98059, LY294002 and rapamycin.

In conclusion, our findings provide important insights into the roles and pharmacological efficacy of BK in regulation of angiogenesis. However, further investigation on the development of BK for the treatment of a variety of diseases associated with angiogenesis should be conducted.

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