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Recombinant Human Kallistatin Inhibits Angiogenesis by Blocking VEGF Signaling Pathway

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ABSTRACT

Kallistatin has been recognized as an endogenous angiogenic inhibitor. However, the underlying molecular mechanism remains poorly understood. Taking it into account that vascular endothelial growth factor (VEGF) has been implicated in all aspects of normal and pathological vasculogenesis and angiogenesis. In this study, we investigated whether VEGF signaling pathway was impacted by the anti-angiogenic effect of recombinant human kallistatin (rhKal). We found that the rhKal inhibited proliferation as well as induced apoptosis of cultured human umbilical vein endothelial cells (HUVECs) in both concentration- and time-dependent manners. The rhKal also suppressed the VEGF-induced migration and tube formation of HUVECs. Furthermore, our data revealed that the rhKal suppressed the VEGF165-stimulated tyrosine phosphorylation of VEGFR-2 as well as its downstream signal molecular activation. The inhibition of receptor phosphorylation was correlated with a decrease in VEGF-triggered phosphorylation of angiogenesis signal molecules AKT and ERK, but not stress-related JNK. Taken together, these findings added the knowledge for us to understand the anti-angiogenic mechanism of kallistatin, which suggested that the rhKal could be worth as a candidate compound for further development for the purpose of anti-angiogenic therapies. J. Cell. Biochem. 115: 575–584, 2014.

KEY WORDS: KALLISTATIN; VEGF; ANGIOGENESIS

Blood vessel formation develops through two different processes, namely vasculogenesis angiogenesis [Demir et al., 2007]. The process of vasculogenesis involves the de novo formation of blood vessels from precursor cells, whereas angiogenesis involves the creation of new vessels from already existing ones [Charnock-Jones et al., 2004]. Commonly, the angiogenesis plays a key role in the tumor's blood vessel formation. Angiogenesis is a fundamental step in physiologic processes, such as organ growth, wound healing, and reproduction, also in pathological conditions like chronic inflammation, arthritis, tumor progression, and metastasis [Folkman, 1995; Risau, 1997]. Tumor growth cannot be independent from blood supply. The process of tumor angiogenesis involves recruitment of sprouting vessels from exciting blood vessels and incorporation of endothelial progenitors into the growing vascular bed [Rafii et al., 2002]. Pathological angiogenesis represents a crucial step in cancer development, where the new blood vessels provides essential oxygen and nutrients to tumors >2 mm in size [Folkman, 1971, 2002]. The angiogenic process is a highly dynamic, and regulated by a lot of mediators including both pro- and anti-angiogenic factors.

Although numerous pro-angiogenic factors have been identified as potential mediators of the angiogenic switch, the vascular endothelial growth factor (VEGF) was reported to be the most significant among them [Lyons et al., 2010]. The human VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. VEGF-A is generated as multiple isoforms by alternative splicing. These isoforms include VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206. VEGF165 is the most predominant form. VEGF signaling is required for the full execution of vasculogenesis

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and angiogenesis. In tumor, the activity of vascular endothelial cell plays a critical role in pathological process. Different types of VEGF binds to three highly related receptors: VEGFR-1 (Flt-1, R1), VEGFR-2 (kdr/Flk-1, R2), and VEGFR-3 (Flt-4, R3), respectively. VEGFR-1 participates in pathological angiogenesis, but its signaling pathway is still unclear. Although the binding of VEGF to VEGFR-1 resulted in increasing production of matrix metalloproteinase-9 and other tissue-specific growth factors [Neufeld et al., 1999], it was not associated with proliferation of endothelial cell [Shibuya et al., 1990; Terman et al., 1992]. The VEGFR-3 receptor is involved in lymph angiogenesis and is not considered to play a critical role in endothelial cell maturation or proliferation. The VEGFR-3 can be activated by VEGF-C and VEGF-D but not VEGF-A. Of these receptors, VEGFR-2 is thought to be the most critical receptor in the regulation of angiogenesis, and its activation is considered essential in endothelial cell proliferation and survival. VEGFR-2 plays a key role to the activation of major downstream effectors responsible for endothelial cell proliferation, migration, differentiation, survival as well as embryonic angiogenesis [Rho et al., 2012]. VEGF-A induces the tyrosine phosphorylation of VEGFR-2 and consequently activate the downstream signaling pathways including AKT (also known as Protein Kinase B) and ERK (extracellular-signal-regulated kinases) [Tournier et al., 2000; Lee et al., 2005].

In the area of anti-cancer research, anti-angiogenesis has been recognized as an important topic. There have been increasingly demands of angiostatic agents in the clinical management of cancer. By exploiting the effective agents, kallistatin has attracted our attention because of its effect on inhibition of VEGF165. Kallistatin is a member of the serine proteinase inhibitor (serpin) [Chai et al., 1993]. Kallistatin rapidly binds to tissue kallikrein therefore inhibits its enzymatic activity in vitro [Zhou et al., 1992]. Kallistatin has been demonstrated a variety of bioactivities in physiologic and pathologic responses, such as anti-inflammation, anti-angiogenesis, and blood pressure regulation [Chen et al., 1997; Miao et al., 2002; Wang et al., 2005; Lu et al., 2007; Zhu et al., 2007]. Recently, many studies have shown that kallistatin significantly inhibit tumor vessel formation. However, the mechanisms remain poorly understood although kallistatin may inhibit the expression of VEGF, or inhibit both expression and activation of kallikrein [Wolf et al., 2001; Lu et al., 2007; Zhu et al., 2007].

In the present study, we produced biologically active recombinant human kallistatin (rhKal) from *P. pastoris* strain GS115 and investigated its potential anti-angiogenic effects both in vitro and in vivo. The results showed that the rhKal inhibited human umbilical vein endothelial cells (HUVECs) growth and migration at cell level, and also inhibited the phosphorylation of VEGFR-2 and its downstream signaling pathways at molecular level. The inhibition of receptor phosphorylation was correlated with a decrease in VEGF165-triggered phosphorylated forms of AKT and ERK.

MATERIALS AND METHODS

MATERIALS

Matrigel was purchased from BD Biosciences; 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and VEGF165 were purchased from Sigma (US); Polyclonal antibodies against Akt and ERK were purchased from Boster Biological Technology, Inc. (Wuhan, China); polyclonal antibodies against phospho- ERK1/2, phospho-Akt, phospho-JNK were obtained from KeyGEN Biotech (Nanjing, China); VEGFR-2, p-VEGFR-2 antibody, horseradish peroxidase (HRP)- conjugated anti-rabbit immunoglobulin (IgG) were purchased from Santa Cruz Biotechnology (SantaCruz, CA).The mouse were purchased from Xiamen university.

EXPRESSION AND PURIFICATION OF RECOMBINANT HUMAN KALLISTATIN

Human kallistatin cDNA was amplified from pAAV-Kal [Tse et al., 2008] (Hua-qiao University) and inserted into pPIC9 vector to generate a recombinant vector of pPIC9- Kal. The pPIC9-Kal vector was transformed into *Pichia pastoris* strain GS115 (His4) by electroporation. The positive strain was cultured in BMMY medium (pH 7.0) at 29°C. The target protein was induced by 2% methanol after 96 hours. Recombinant human kallistatin (RhKal) protein was purified from the supernatant with Heparin Sepharose FF chromatograph and Phenyl Superose (GE) successively. Expression of rhKal was confirmed by SDS-PAGE and Western-blotting.

THE LIPOPOLYSACCHARIDES ASSAY

The chromogenic end-point tachypleus amebocyte lysate (Horseshoe Crab Reagent Manufactory, XiaMen, China) kit was applied to test the lipopolysaccharides (LPS) concentration in the preparations of rhKal following the manufaturer's instruction.

CELL CULTURE AND VIABILITY ASSAY

HUVECs were cultured in 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), and maintained in a humidified atmosphere of 5% CO_2 at 37°C two passages weekly. Cell proliferation was assessed by MTT staining. Cells (8×10^3) were incubated in 96-well plates under various concentrations of rhKal as indicated. After 24, 48, 72 h of incubation, six wells were selected from each group of cells for MTT (50 mg/well) assay. After incubation for 4 h, the reaction was stopped by adding 150 μl/well of DMSO, and allowed a further incubation for 10 min. The color reaction was quantified using an automatic plate reader (Molecular Devices) at 570 nm. Cell viability was determined by the colorimetric quantification.

CELL PROLIFERATION ASSAY

HUVECs (3×10^5) were seeded in 6-well plates for 24 h, and then treated with the rhKal at the final concentrations of 25, 50, and 100 μg/ml, respectively, for another 48 h. In parallel, we also measured the effect of LPS (1 EU/ml) on the proliferation of HUVEC. The cells were collected and washed twice in cold PBS, and resuspended in 300 μl PBS at room temperature. Seven hundred microliters of cool ethanol was added to the cells for 1h at 4°C. The cells were centrifuged and incubated with 0.5 ml HCl (2 N) with 0.5% Triton-X-100 for 30 min. After that, the cells were centrifuged and incubated 0.5 ml $Na_2B_4O_7$ (0.1 M) for 2 min. The cells were collected and washed twice in cold PBS, and resuspended in 50 μl PBS containing 0.5% Tween-20, 1% BSA/PBS, and 20 μl mouse anti-Ki67 antibody and then incubated at room temperature for 1 h. After washing with 150 μl PBS containing 1% BSA, 5 μl anti-mouse secondary antibody (1:1,000) were correspondly added and incubated

at 37°C for 30 min. Finally, 500 μl of cold PBS was added to each tube and analyzed by flow cytometry (Epics xl, BECKMAN).

CELL APOPTOSIS ANALYSIS BY FLOW CYTOMETRY

HUVECs (3×10^5) were seeded in six-well plates for 24 h, and then treated with the rhKal at the final concentrations of 0.2, 25, 50, and 100 μg/ml, respectively, for another 48 h. The cells were collected and washed twice in cold PBS, and resuspended in100 μl PBS at room temperature. The suspension was incubated with 5 μl propidium iodide (20 μg/ml) and 5 μl annexin V-FITC (1 μg/μl) in dark for 30 min at room temperature (both reagents from Key GEN, Nanjing, China). Finally, 400 μl of cold PBS was added to each tube and analyzed by flow cytometry (Epics xl, BECKMAN). Calculation of apoptosis rate (AR): AR (%) = (NSC − NVC)/NSC, where NSC = events corresponding to seeded cells; and NVC = events corresponding to viable cells after culture [number of annexin V(−) cells].

ENDOTHELIAL CELL MIGRATION ASSAY

Scratch-wound directional migration assay. The cells migration was detected using a two-dimension (2D) model. HUVECs were seeded at cell density of (2×10^5 cells/well) in a six-well plate. The cells were cultured for 24 h and allowed to growth into a confluent monolayer. The cell monolayer was wounded with a small cell scraper. Thereby a 2D detection zone with approximate 1 mm width for observation of cell migration was created. After washing with PBS three times, the cells were incubated with a new medium containing VEGF165 (10 ng/ml) and various concentration of rhKal (0, 20, 40, and 60 g/ml, respectively). In parallel, we also measured the effect of LPS (1 EU/ml) on the migration of HUVECs. The cells migration from the both edges of the scratch was observed and photographed under a microscope (Nikon, Japan).

Transwell cell migration assay. Chemotactic motility of HUVECs was measured using a Transwell (8-μm pores; BD Biosciences) according to the manufacturer's instructions. Briefly, the polycarbonate filter between the lower and upper chambers was coated with 0.2% gelatin at 4°C overnight and then air-dried. The lower chamber was filled with a 30 μl aliquot of basal 1640 medium containing 0.2% BSA with rhKal (0, 20, 40, and 60 μg/ml, respectively) and VEGF165 (10 ng/ml). Then 3×10^5 cells suspended in the medium with neither rhKal nor VEGF were loaded in the upper chamber. After 4 h at 37°C with 5% CO_2, the filter was fixed and stained with Gimesa staining. Non-migrating cells on the upper surface of the filter were discarded. Chemotaxis of the cells was quantified by counting the number of cells that migrated into filter under an optical microscope (100×, Nikon, Tokyo, Japan). Six fields evenly crossed each filter were counted. The distance of the wound edges was measured and calculated by Image J. All data were analysis by Dunnett's test. Experiments were performed in triplicates.

TUBE FORMATION ASSAY

Tube formation assay was performed as described previously [Huh et al., 2010]. Briefly, a 96-well plate was coated with 60 μl of matrigel (BD Biosciences), which was allowed to polymerize and solidify at 37°C for 1 h. The HUVECs incubated in six-well plate with 1640 medium containing 1% FBS for 6 h were treated with trypsin before harvest. The cells were suspended in 1640 medium containing 1% FBS and aliquotated. Various concentrations of rhKal (50, 100, 200 ng/ml) were added to the cells for 60 min before seeding onto the matrigel layer and then VEGF165 (10 ng/ml, Final) was added. After 18 h, blood-vessel-like tubules from six randomly chosen fields were counted and photographed under a microscope (Nikon).

MICE MATRIGEL PLUG ANGIOGENESIS ASSAY

The Matrigel assay was performed as described previously [Huh et al., 2010]. Briefly, 6-week-old BABL/c mice were subcutaneously injected with 0.5 ml of growth factor-reduced Matrigel containing VEGF165 (150 ng/ml) without or with rhKal (150 μg/ml and 150 ng/ml). On Day 8, the mice were sacrificed. The Matrigel plugs were collected and fixed. The plugs were embedded in paraffin and sectioned. The sections were stained with Masson's trichrome solution containing iron hematoxylin, acid fuchsin, and light green dye. The solution stained the Matrigel blue; erythrocytes/vessels red; and nucleated blood cells brown, which was very useful for assessment of neovascularization. Procedures of the animal experiments were approved by Animal Care & Ethics Committee, The School of Biomedical Sciences, Huaqiao University.

WESTERN BLOTTING

HUVECs (3×10^5) were seeded in six-well plates for 24 h and then were treated with rhKal for different durations (6, 12, 24, 48 h) in concentrations (50, 100, 200 μg/ml). All the HUVECs from each sample treated were collected and lysed for 15 min in pre-cold lysis buffer containing 29 mM Tris–HCl, pH 7.4, 137 mM NaCl, 10% (w/v) glycerin, 1% (v/v) Triton X-100, 2 mM EDTA, 1 mM PMSF). Cell debris was removed by centrifugation at 16,000*g* for15 min. Protein concentration of each supernatant was determined using a BCA Protein Assay Kit (Thermo Scientific). Equal amounts of proteins were separated on a 12% SDS-PAGE, The separated protein bands were transferred electrophoretically (Bio-Rad) onto a nitrocellulose membrane (KeyGEN, Nanjing, China). The membrane was blocked with 5% non-fat milk powder (w/v) in tris-buffer saline Tween-20 (TBST, 10 mM Tris, 100 mM NaCl, and 0.1% Tween-20) for 2 h at room temperature. The membranes were incubated with primary rabbit anti-human kallistatin polyclonal antibodies or mouse anti-human kallistatin monoclonal antibodies (1:500) overnight at 4°C, and with rabbit anti-human β-actin polyclonal antibody (1:500) as a control. After washing with TBST five times, HRP-conjugated anti-rabbit or mouse secondary antibody (1:1,000) were added corresponding with the primary antibodies and incubated at 37°C for 1 h before another five-time washing. Labeled bands were developed with enhanced chemiluminescence detection system (Amersham Pharmacia, UK) and photographed with a Molecular Imager Gel Doc XR system (Bio-Rad). Targeted protein levels were quantified by density analysis using Quantity One software (Bio-Rad). Relative protein expression levels were deduced from the ratio of the mean values of each band to that of β-actin.

STATISTICAL ANALYSIS

Assays were performed in duplicate and three independent experiments unless otherwise stated. Statistically significant differences between control and experimental groups were calculated by Dunnett's test. Statistically significant is expressed as $^{*}P < 0.05$, while highly statistically significant as $^{**}P < 0.01$.

RESULTS

EXPRESSION AND PURIFICATION OF rhKal

The rhKal was expressed in *P. pastoris* strain GS115 and purified by Heparin Sepharose FF and Phenyl Superose chromatograph. The process of purification was monitored by SDS-PAGE (Fig. 1A). The identity of the protein was confirmed by Western blotting analysis using an antibody against human kallistatin. The molecular weight of the glycosylated protein was 44–58 KD on SDS-PAGE (Fig. 1B). An average of 20 mg of purified rhKal in soluble form was obtained from 1 L of the culture.

THE LIPOPOLYSACCHARIDES ASSAY OF rhKal

Chromogenic end-point tachypleus amebocyte lysate kit was used to test the potential contamination of LPS in the rhKal preparations. A series of concentrations of standard LPS (0.0625, 0.125, 0.25, 0.5, 1.0 EU/ml) were used to make a standard curve. An equation of regression was established based on the standard curve. $Y = 0.556 X + 0.019$. The highest concentration of rhKal in the preparations for this study was 200 μg/ml. The absorbance of rhKal sample (200 μg/ml) was 0.457. So the concentration of LPS in rhKal (200 μg/ml) is 0.788 EU/ml.

THE rhKal INHIBITED VIABILITY OF HUVECs

The HUVECs were cultured in presence of rhKal at a range of concentrations (40–640 μg/ml in final) for different durations (24–72 h). The experiment included a group of cultured without rhKal as negative control. The proliferation inhibition rates were calculated from the loss of cell viability by assessment with MTT assay. The rhKal inhibited the growth of HUVECs in dose- and time-dependent manners (Fig. 2). The median inhibition concentration (IC50) values for 48 h, and 72 h were 189.6 μg/ml and 146.5 μg/ml, respectively.

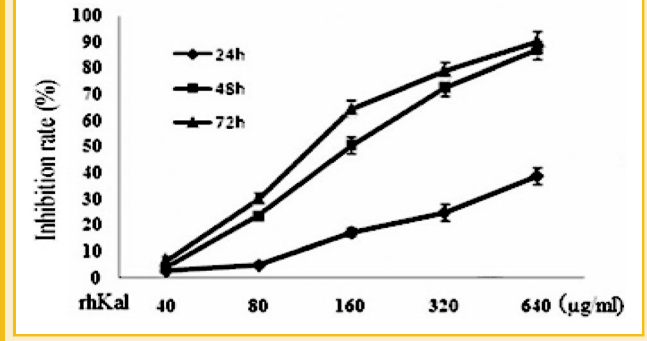

Fig. 2. Effect of rhKal on cell viability in HUVECs. The rhKal inhibited the growth of HUVECs in dose- and time-dependent manners.

THE rhKal AFFECTS THE CELL PROLIFERATION

Based on the viability results above, it would be interesting to know the anti-proliferation effect of rhKal. We selected doses of 0.2 ng/ml, 25, 50, and 100 μg/ml to determine the inhibitory effects of proliferation by flow cytometry. In parallel, we also measured the effect of LPS (1 EU/ml) on the proliferation of the HUVECs. The proliferation rates were 94.3%, 94%, 93.6%, 93.2%, 89.8%, 88.5%, respectively. The results showed that the rhKal was not capable of inhibition of the cell proliferation (Fig. 3).

THE rhKal INDUCED APOPTOSIS OF HUVECs

Based on the viability and proliferation effect on the HUVECs, we selected doses of 200 ng/ml, 25, 50, and 100 μg/ml to determine the inhibitory effects of apoptosis by flow cytometry. The apoptosis rates were 1.04%, 1.6%, 3.9%, 6.7%, 16.2%, respectively. The results showed that the rhKal induced apoptosis in a concentration-dependent manner (Fig. 4). So we supposed the inhibition of the growth on HUVECs may be related with the pro-apoptosis of rhKal.

THE rhKal SUPPRESSED VEGF- INDUCED MIGRATION OF HUVECs

The effect of kallistatin on chemo tactic motility of HUVECs was measured using two-dimensional migration assay and Transwell

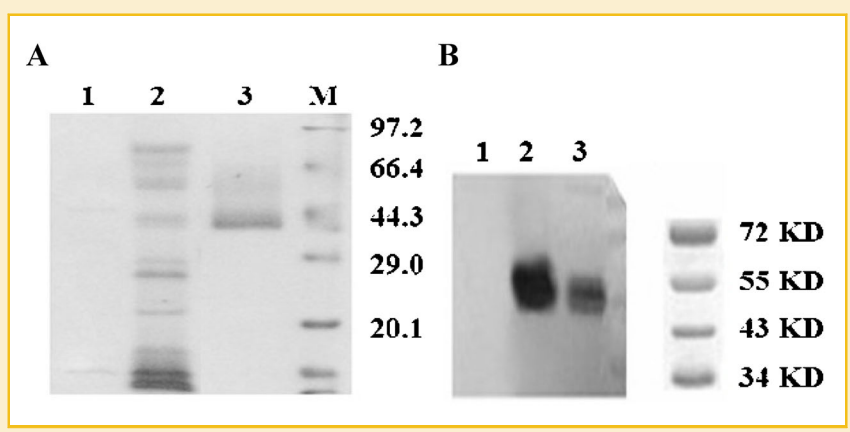

Fig. 1. Characterization of expressed rhKal by SDS-PAGE and western blotting. A: Coomassie blue staining of polyacrymide gel. Lane 1: Penetration peak washed with balanced solution; lane 2: washed with 0.1 mol/L NaCl; lane 3: Eluted with 0.5 mol/L NaCl; lane M: protein marker molecular weights are indicated on the right site. B: Western blot analysis using specific antibody. Lane 1: the fermentation broth of wide strain; lane 2: sample of rhKal 1; lane 3: sample of rhKal 2.

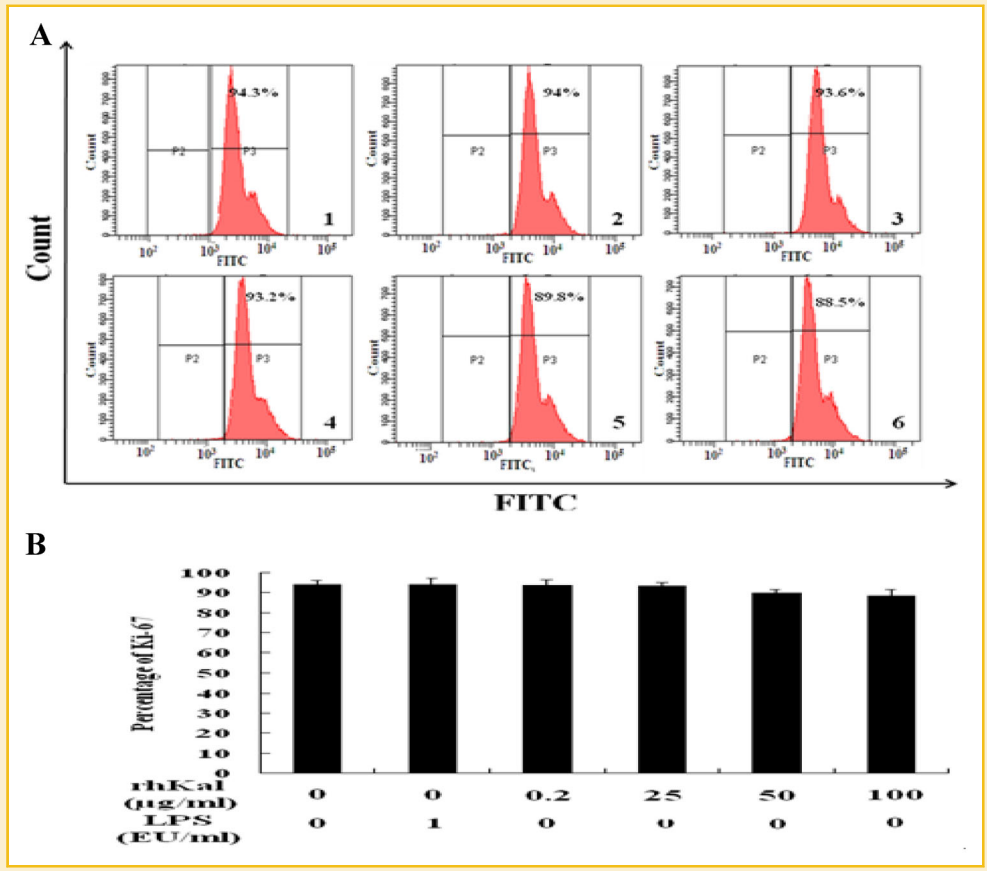

Fig. 3. Effect of the rhKal on cell proliferation in HUVECs. A: The rhKal has not effect on the proliferation of HUVECs. (1: Control; 2: 1 EU/ml LPS; 3: 200 ng/ml rhKal; 4: 25 μg/ml rhKal; 5: 50 μg/ml rhKal; 6: 100 μg/ml rhKal); (B) The quantitative results of flow cytometry analysis (n = 3).

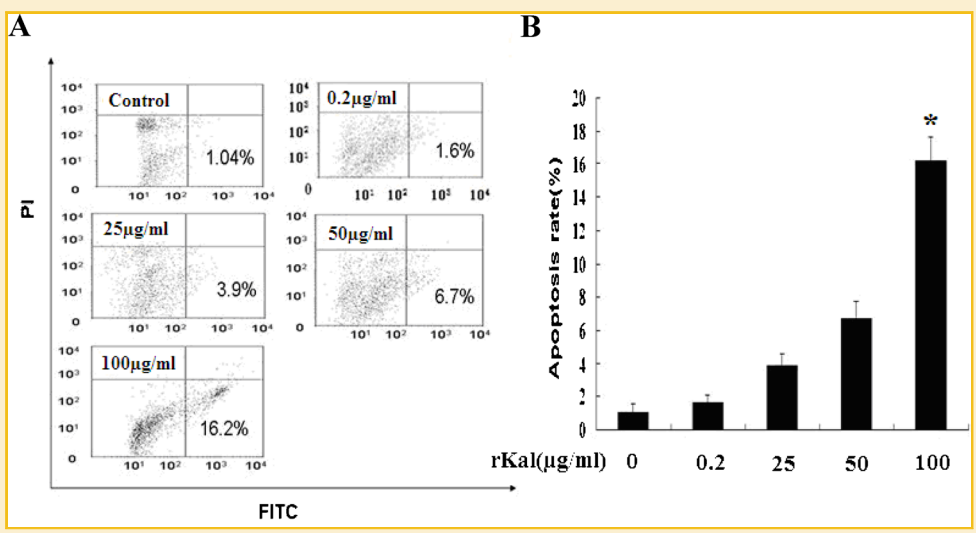

Fig. 4. Effect of the rhKal on cell apoptosis in HUVECs. A: The rhKal caused strong apoptosis in the HUVECs after treated with a series of concentrations of the rhKal for 48 h. The cells were collected and stained with annexin V-FITC/PI. (1: Control; 2: 0.2 μg/ml rhKal; 3: 25 μg/ml rhKal; 4: 50 μg/ml rhKal; 5: 100 μg/ml rhKal); (B) The quantitative results of flow cytometry analysis. $^{*}P < 0.05$ vs. control group, n = 3.

chamber assay. In the two-dimensional migration assay, the migration of the HUVECs were substantially inhibited after treatment with a range of concentrations (20–60 μg/ml) of the rhKal for different durations (0–36 h), compared with the VEGF165 group and blank control (Fig. 5). In the Transwell model, after stimulating the HUVECs with VEGF165 (10 ng/ml) for 4h, more number of HUVECs migrated to the lower side of the membrane in the Transwell chamber than the group treated with the rhKal. The rhKal significantly inhibited VEGF165-induced migration of HUVECs in a concentration-dependent manner (Fig. 6). The above results demonstrated that the rhKal has an ability to inhibit VEGF165-induced migration of HUVECs in vitro.

THE rhKal SUPPRESSED VEGF165-INDUCED TUBE FORMATION of HUVECs

The HUVECs on matrigel were stimulated with VEGF165 which promoted differentiation of the cells into a tube form (Fig. 7A2). The rhKal effectively reduced the quantity of endothelial tubes induced by VEGF165 in a concentration-dependent manner (Fig. 7A1–5). The highest concentration (200 ng/ml) of rhKal either inhibited proliferation or induced apoptosis of HUVECs. Quantitative analysis showed that treatment with the rhKal resulted in 89%, 67%, and 45% inhibition of VEGF165-stimulated tube formation at 10 ng/ml (Fig. 7B). The results demonstrated that rhKal had the ability to block VEGF165-induced tube formation of HUVECs.

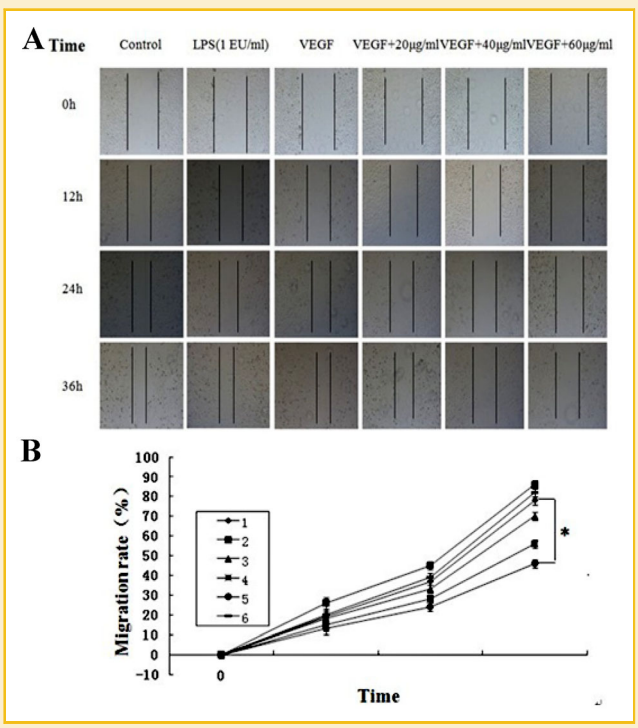

Fig. 5. The rhKal inhibited VEGF-induced migration of HUVECs in a scratch-wound model. The inhibition was dose- and time- dependent. A: 2D images with inserted lines indicated the wound edges; (B) Graphic illustration reflecting the migration rates of each group (1: Control; 2: 1 EU/ml LPS; 3:10 ng/ml VEGF165 and 0 μg/ml rhKal; 4: 10 ng/ml VEGF165 and 20 μg/ml rhKal; 5: 10 ng/ml VEGF165 and 40 μg/ml rhKal; 6: 10 ng/ml VEGF165 and 60 μg/ml rhKal).

THE rhKal INHIBITS VEGF165-INDUCED MATRIGEL PLUG IN VIVO ANGIOGENESIS

The anti-angiogenesis activity of the rhKal was accessed by an in vivo test, Matrigel plug assay. The plugs removed from the experimental animals were sectioned and stained with Masson′s trichrome solution. The solution stained the Matrigel in blue, erythrocytes/vessels in red, and nucleated blood cells in brown. It showed that rhKal strongly inhibited VEGF165-stimulated angiogenesis. Masson stain revealed that the higher dose of rhKal significantly decreased the red cells in Matrigel compared with VEGF165-stimulated Matrigel (Fig. 8). The results suggested that the rhKal effectively inhibited in vivo angiogenesis induced by VEGF165.

INFLUENCE OF THE rhKal ON VEGFR-2 AND RELATED SIGNALING PATHWAYS

In this part, we investigated the effects of rhKal on the VEGF165-stimulated activation of VEGFR-2 as well as the down-stream signaling molecules AKT, ERK1/2 and JNK in HUVECs. As shown in Figure 9, exogenous rhKal apparently repressed phosphorylation of VEGFR-2. Pre-treatment with the rhKal suppressed VEGF165-stimulated phosphorylation of VEGFR-2 without affecting the over all levels of VEGFR-2 expression. Treatment with the rhKal also efficiently inhibited phosphorylation of Akt and ERK1/2 activated by VEGF165 in concentration- and time-dependent manners without JNK (Fig. 9). With the increase of concentration and duration, the inhibition rate was also raising. In all the experimental conditions, the total steady-state protein levels of AKT, and VEGFR-2 remained unchanged, except for ERK1/2. All the results suggest that VEGFR-2 may be a critical target blocked by rhKal leading to interruption VEGF165-induced signal transduction.

DISCUSSION

Angiogenesis, the process leading to the formation of new vessels, involved a series of complex steps including migration, proliferation, and capillary tube formation of endothelial cells [Carmeliet et al., 1996; Patan, 2000]. In the first phase, the expression of angiogenic stimulator cytokines will be significantly enhanced. The quantitative balance between angiogenic stimulator and anti-angiogenic cytokines should be broken. The second phase occurs when activated endothelial cells start to proliferate and migrate towards forming a vascular sprout. In the third phase, the vascular sprout forms a lumen. Lastly, pericytes are recruited to the area to add up support for the new vessel [Leung et al., 1989; Ferrara and Davis-Smyth, 1997]. There are a lot of other types of cells and molecules involved in the whole process. Among the endogenous angiogenic mediators, VEGF is the most specific and potent angiogenic stimulator and generated by almost all tumor cells [Ferrara et al., 2003; Pan et al., 2011].

It has been demonstrated that the rhKal inhibited HUVECs viability with a time- and dose-dependent manner but no effect on proliferation. The rhKal induced HUVECs apoptosis effectively in a dose-dependent manner by flow cytometry analysis. So, we supposed the inhibition of the growth of HUVECs may be related with the pro-apoptosis of rhKal. At the same time, Wound healing assay and Transwell chamber indicated that rhKal inhibited the migration of

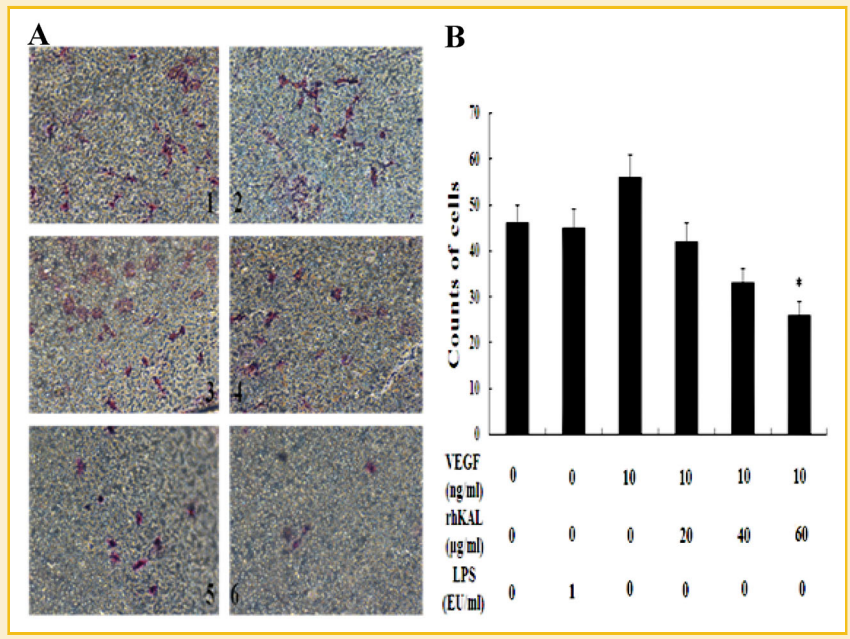

Fig. 6. The rhKal inhibited VEGF-induced migration of HUVECs in transwell chamber assay. (A) 1: Treated with neither VEGF nor rhKal as a control; 2: Treatment with 1 EU/ml LPS; 3: 10 ng/ml VEGF165; 4: 10 ng/ml VEGF165 and 20 μg/ml rhKal; 5: 10 ng/ml VEGF165 and 40 μg/ml rhKal; 6: 10 ng/ml VEGF165 and 60 μg/ml rhKal. Arrows point (cells). B: Illustration of cell numbers who have migrated from upper chamber to the lower side of the membrane between the upper and lower chambers. The inhibition was dose dependent. Error bars indicate standard deviation (SD). $^{*}P < 0.05$.

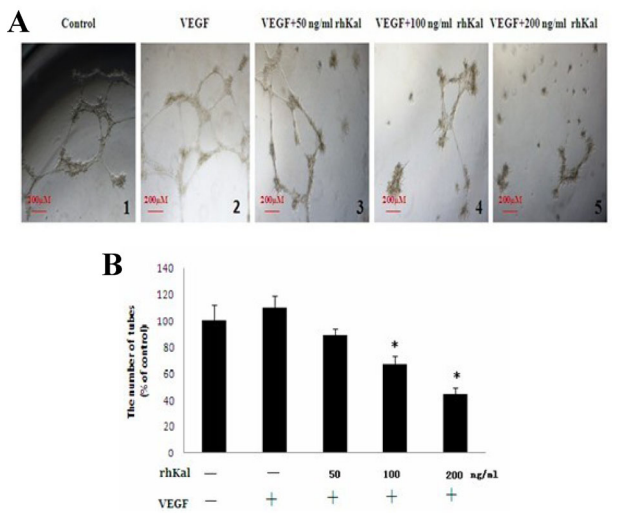

Fig. 7. Effect of rhKal on VEGF-induced tube formation of HUVECs. A: (1) Control; (2)Treatment with 10 ng/ml VEGF165; (3) Treatment with 10 ng/ml VEGF165 and 50 ng/ml rhKal; (4) Treatment with 10 ng/ml VEGF165 and 100 ng/ml rhKal; (5) Treatment with 10 ng/ml VEGF165 and 200 ng/ml rhKal. B: The quantitative results of tube formation analysis. $^{*}P < 0.05$ vs. control group, n = 3.

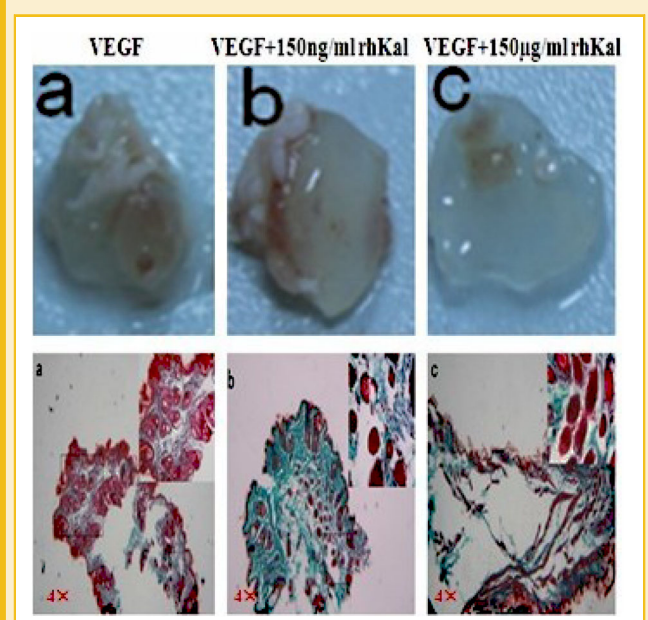

Fig. 8. The rhKal suppressed VEGF-induced angiogenesis in vivo Matrigel Plug Angiogenesis Assay. a: The Matrigel was mixed with VEGF165 (150 ng/ml) alone; (b) The Matrigel was mixed with VEGF165 (150 ng/ml) and 150 ng/ml rhKal; (c) The Matrigel was mixed with VEGF165 (150 ng/ml) and 150 μg/ml rhKal. Top panels: whole view of each of the plug under microscope [1 × 1]; bottom panels: Masson's trichrome solution stained sections with magnification of [4 × 10] from each plug with insertions [40 × 10]. Colors in blue: Matrigel; red: erythrocyte; brown: nucleated blood cells. The results showed that the rhKal effectively inhibited the erythrocyte and angiogenesis.

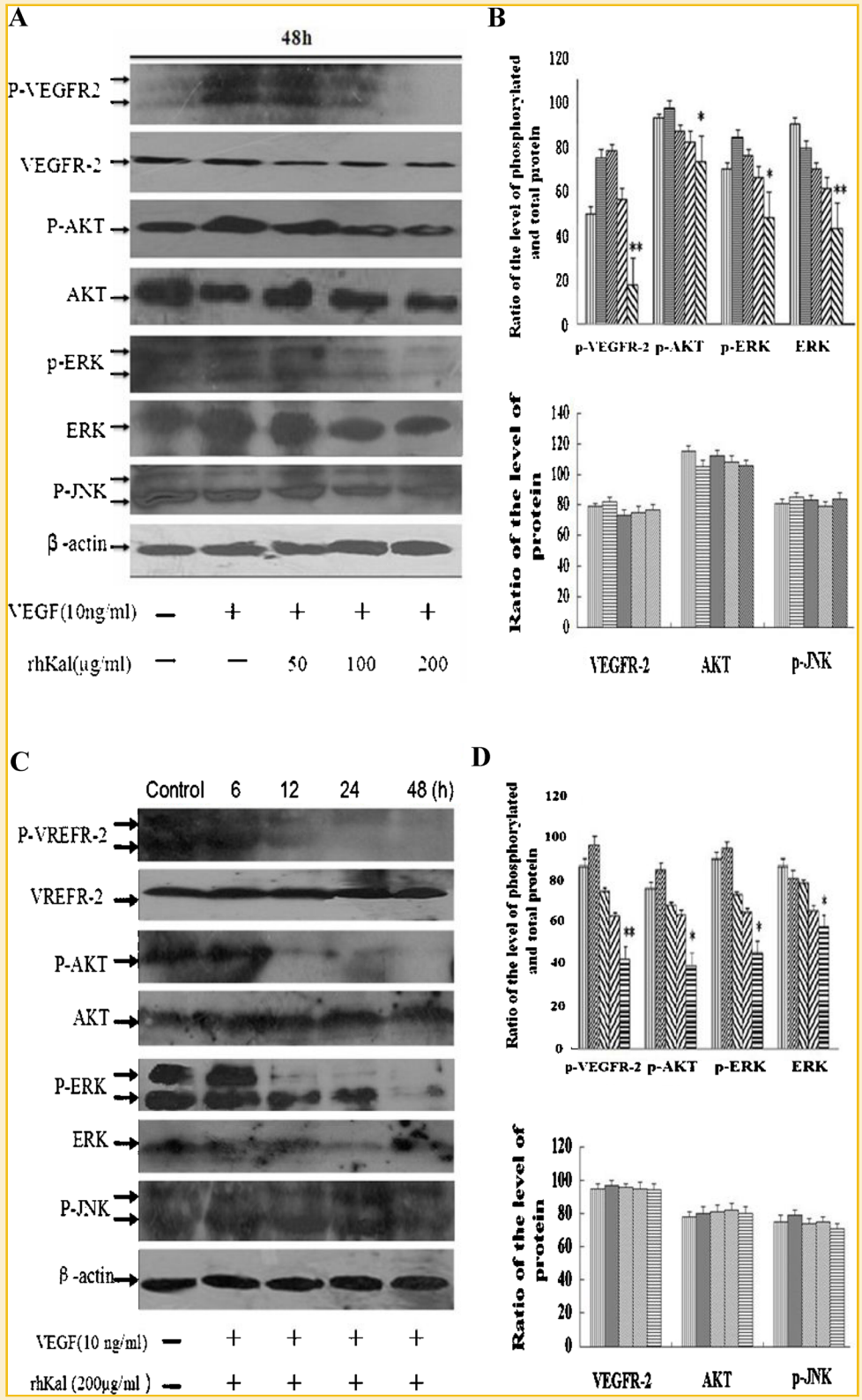

Fig. 9. Effect of rhKal on VEGF-induced activation of VEGFR-2 and related signaling pathways. A: Western blot were used to compare phosphorylated and total of VEGFR2, AKT, ERK1/2, JNK after treated with various dose of rhKal for 48 h.The quantitative results of related proteins were analyzed after treatment with rhKal. B: The quantitative results of related proteins after treatment with rhKal for 48 h. The data are expressed as mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group. C: Western blot were used to compare total and phosphorylated VEGFR2, AKT, ERK1/2, JNK after treated with 200 μg/ml rhKal for 6, 12, 24, 48 h, respectively. D: The quantitative results of related proteins after treatment with rhKal for for 6, 12, 24, 48 h, respectively. The data are expressed as mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

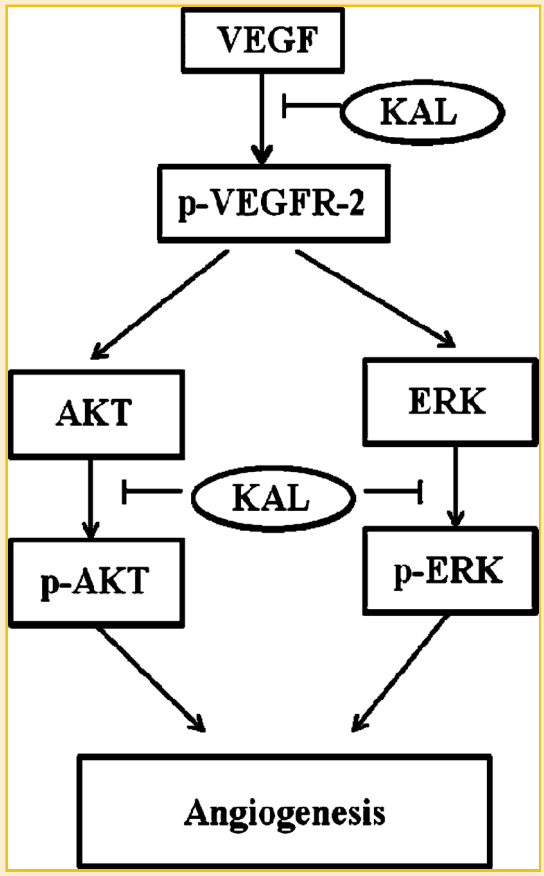

Fig. 10. The proposed interact sites of kallistatin along VEGF angiogenesis signaling pathway.

HUVECs in response to VEGF165 stimulation in vitro, using the concentrations that did not suppressed their growth in these experiments. Differentiation and rearrangement of endothelial cells leads a vascular tube formation, which is a critical step of angiogenesis [Hanahan et al., 1996]. Furthermore, the rhKal directly reduced VEGF165-induced tube and vessel formation by HUVECs on marrigel in vitro and in vivo. All the above results indicated that rhKal could act as a potential anti-angiogenic protein.

The biological activity of VEGF has been well documented. VEGF has been implicated in all aspects of pathological vascular-endothelial-cell biology. VEGF binds to three highly related receptors: VEGFR-1 (Flt-1, R1), VEGFR-2 (kdr/Flk-1, R2), and VEGFR-3 (Flt-4, R3). Of these receptors, VEGFR-2 is thought to be the most critical receptor in the regulation of angiogenesis, and its activation is considered essential in endothelial cell proliferation and survival. It is a member of the receptor tyrosine kinase family expressing on the surface of endothelial cells [Lyons et al., 2010]. Its expression levels are remarkably increased in the pathologic states, such as neo-vascularization occurs [Neufeld et al., 1999]. VEGF can induce the tyrosine phosphorylation of VEGFR-2 and active the downstream signaling pathways, such as that through the phosphatidylinositol 3-kinase/AKT and the mitogen-activated protein kinase (MAPK) superfamily (including ERK1/2, JNK, and p38 MAPK) [Tournier et al., 2000; Lee et al., 2005]. As downstream signal protein of VEGFR-2, AKT plays an important role on VEGF-induced angiogenesis by stimulating the phosphorylation of nitric oxide synthase (eNOS) [Six et al., 2002]. Binding of VEGF and VEGFR-2 induces receptor tyrosine phosphorylation and stimulates the phospholipase Cγ-protein kinase C-MAPK pathway [Zachary and Gliki, 2001]. It has been suggested that ERK signaling pathway is preferentially activated in response to growth factors, and is generally known to promote the proliferation, migration, and morphogenesis of endothelial cells [Keshet and Seger, 2010]. The JNK family belongs to the mitogen-activated protein kinase (MAPK) superfamily, downstream single molecule of VEGFR-2, involved in cell proliferation, apoptosis, and angiogenesis [Ishikawa and Kitamura, 1999].

In contrast to the effect of kallistatin on angiogenesis, the mechanism of the anti-angiogenic activity is still not well understood. Based on the above findings that the rhKal blocked VEGF165-induced angiogenesis both in vitro and in vivo, we tested the hypothesis that its molecular mechanism may involve an approach through the interaction with VEGF165 and VEGFR-2. By investigating the interaction between VEGF165 and VEGFR-2, we have found that the rhKal effectively suppressed VEGF165-induced phosphorylation of VEGFR-2, but did not affect the expression levels of total VEGFR-2 proteins. We have also found that the rhKal effectively inhibited the phosphorylation of Akt, ERK1/2, but not JNK induced by VEGF165. JNK (c-Junc-Jun N-terminal kinase) is responsive to stress stimuli although it also belongs to the MAPK family. It also can be activated by VEGF-VEGFR-2 pathway. In our study, it did not affect the total proteins levels of observed proteins along the singling pathway, respectively, except ERK1/2. The rhKal may inhibit the total protein and phosphorylation of ERK1/2 at the same time. The inhibitory effects expressed in a time- and dose-dependent manner. The results revealed that the inhibitory effect of the rhKal on VEGFR-2 and the downstream signaling molecular may be, at least in part, contribute to its anti-angiogenic activity (Fig. 10).

In summary, this study demonstrated that the rhKal has potential anti-angiogenic activity both in vitro and in vivo. The mechanism of the anti-angiogenic activity was evidenced involving interruption of VEGF165-induced phosphorylation of VEGFR-2 and subsequent inhibition of VEGF165-stimulated phosphorylation of Akt and ERK. Collectively, these findings reinforce the notion that it is worth further investigation of kallistatin for the purpose of anti-angiogenic therapies.

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