Anti-angiogenic action of plasma hyaluronan binding protein in human umbilical vein endothelial cells

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Abstract. The kringle domain is a triple loop structure present in angiostatin and endostatin. The disulfide bond-linked kringle architectures have been known to be essential for anti-angiogenic activity. Plasma hyaluronan binding protein (PHBP) is a novel serine protease which consists of three epidermal growth factor (EGF) domains, a kringle domain, and a serine protease domain. PHBP can be cleaved auto-catalytically to generate activity and is highly expressed in the human blood and liver. To determine the anti-angiogenic activities of PHBP, we purified recombinant mouse PHBP from stable cell line overexpressing PHBP and used protein *in vivo* and *in vitro* angiogenesis assays. We found that recombinant PHBP inhibits not only angiogenesis *in vivo* in chorioallantoic membrane (CAM) assay but also the basic fibroblast growth factor (bFGF)-induced proliferation, invasion and tube formation of human umbilical vein endothelial cells (HUVECs) in a dose-dependant manner. Moreover, we found that the kringle domain of PHBP was essential for the anti-angiogenic action of PHBP by the deletion mutants. These findings unravel a new function of PHBP as an inhibitor of the proangiogenic phenotype of vascular endothelial cells and demonstrate that the kringle domain of PHBP might be a potent novel inhibitor of activated endothelial cells *in vitro* and *in vivo*.

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Abbreviations: CAM, chorioallantoic membrane; PHBP, plasma hyaluronan binding protein; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor

Key words: angiogenesis inhibition, plasma hyaluronan binding protein, factor VII activating protease

Introduction

Angiogenesis, the sprouting of new capillaries from pre-existing vasculature, is an essential physiological process in embryonic development, wound healing, and reproductive cycles in adult females (1). It is also recognized as a characteristic of pathological conditions such as psoriasis, proliferative retinopathies, and cancer growth and metastasis (2). During angiogenesis, endothelial cells need to divide, migrate, invade the extracellular matrix, and form capillary structures from pre-existing blood vessels (3). These complex processes imply the presence of multiple controls, which can be temporarily turned on and off within a short period (4,5). A switch of the angiogenic phenotype by an up-regulation of angiogenesis activators and down-regulation of angiogenesis inhibitors often leads to the progression of many diseases (1,6,7).

An endogenous angiogenesis inhibitor, angiostatin, is an internal fragment of plasminogen and contains the first three or four triple loop structures, known as kringle domains (8). The primary amino acid sequence of each kringle domain is composed of approximately 80 amino acids and the structure exists in many proteins. The disulfide bond-linked kringle architectures are essential for the anti-angiogenic activity of angiostatin (9). A previous study shows that kringle fragments of several other proteins also inhibit angiogenesis (8). Most kringles only inhibit angiogenesis when cleaved as fragments from their parental proteins that lack anti-angiogenic activity.

PHBP, isolated by adsorption to immobilized hyaluronan, is a novel glycoprotein found in human plasma. The domain structure of PHBP is composed of one secretion signal peptide, three epidermal growth factor (EGF) domains, one kringle domain and one serine protease domain from its amino-terminus (10). PHBP has the ability of the protease to

activate coagulation factor VII (FVII), named FVII-activating protease (FSAP) (11,12), and to activate tissue- or urinary-plasminogen activators (11,17).

Single-chain PHBP is a 70-kDa zymogen that exists in human plasma at a concentration of 12 μg/ml and can be cleaved autocatalytically to generate the active two-chain form (50 kDa and 27 kDa) linked by a disulfide bond (13,14). PHBP exhibits a strong affinity for negatively charged substances such as hyaluronic acid, dextran sulfate, or heparin, all of which enhance autoactivation. At a cellular level, PHBP interacts with glycosaminoglycans (GAGs) and directly cleaves matrix proteins such as fibronectin, and fibrinogen (17,18). In addition, PHBP specifically binds to vascular smooth muscle cells and reduces platelet-derived growth factor (PDGF)-dependent smooth muscle cell proliferation. The complex formation between PHBP and PDGF has been reported as the major cause (16).

Recently, it has been reported that PHBP inhibits bFGF/EGF-induced proliferation of HUVECs (18). The authors present two possible mechanisms involved. First, PHBP cleaves adhesion molecules which are required for attachment and proliferation. Second, PHBP strongly binds to and partially hydrolyses bFGF. However, even though PHBP affects the proliferation of endothelial cells, the precise mechanisms of PHBP on the process of angiogenesis were not elucidated. Therefore, more detailed studies are required to determine the effects of PHBP on the process of angiogenesis.

In order to determine whether PHBP containing kringle domain can be a novel angiogenesis inhibitor, we performed *in vivo* and *in vitro* angiogenesis assays using recombinant mouse PHBP.

Materials and methods

Cell culture. HUVECs were grown on 0.3% gelatin-coated dishes and maintained in endothelial cell growth medium EGM-2 kit (Clonetics, San Diego, CA). HUVECs were used at passage from 2 to 8. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were grown in a 37˚C incubator with a humidified atmosphere containing 5% CO_2, 95% air.

Construction of the expression vector. The *E. coli* expression vector (pBluescript, Stratagene) of full-length mouse PHBP cDNA was obtained from Dr N.-H. Choi-Miura (Showa University, Japan).

To examine the effects of PHBP on angiogenesis, full-length mouse PHBP cDNA was constructed with the mammalian expression vector, pcDNA3.1/V5-His-TOPO (Invitrogen, Grand Island, NY). The complete open reading frame of PHBP cDNA was produced by PCR from *E. coli* expression vector. The following primers were used: forward, GAGATGTTTGTCAGGATGTTG; and downstream, GAG GCCAGCCTCCCTGT.

Expression and purification of recombinant mouse PHBP. Constructed plasmid DNA was introduced into HEK293 cells using Lipofectamine and Plus reagents (Invitrogen, Carlsbad, CA) and selected in DMEM with 10% FBS and 400 μg/ml G418. The stable cell line was incubated with DMEM containing 1% FBS, and then the conditioned medium was harvested and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-V5 antibody (Invitrogen, Carlsbad, CA). Recombinant mouse PHBP from conditioned medium was concentrated using Vivaspin concentrator (Sartorius, Hannover, Germany), purified using Ni-NTA magnetic agarose beads (Qiagen, Valencia, CA) under native conditions, according to the manufacturer's instructions.

Chorioallantoic membrane (CAM) assay. To determine the anti-angiogenic activity of recombinant mouse PHBP *in vivo*, a CAM assay was performed as previously described (21,22). Fertilized eggs (Pulmuone, Kyungki-do, Korea) were incubated at 37˚C and 90% relative humidity. After 3 days, approximately 2-3 ml of albumin was removed and a window was made. At the 4.5-day-old CAM, test samples or retinoic acid (1 μg) loaded on a quarter size of Thermanox coverslip (Nunc International, Naperville, IL) were applied on the CAM of individual embryos. After 48-h incubation, 10% fat emulsion (Intralipose; Korea Green Cross, Seoul, Korea) was injected into the CAM for observation of the inhibition zone of angiogenesis under a microscope.

[^{3}H]-thymidine incorporation assay. To examine the anti-proliferative effect of PHBP, HUVECs were seeded at a density of $2x10^4$ cells/well in a 24-well plate. Cells were incubated in EGM and allowed to attach for 24 h. Cells were washed two times with endothelial basal medium (EBM) and incubated for 6 h in EBM containing 1% FBS. Cells were stimulated by the addition of the indicated concentration of PHBP and 25 ng/ml of bFGF (Upstate, Lake Placid, NY) for 24 h, and were subjected for 4 h to the addition of 1 μCi/ml [^{3}H]-thymidine (Amersham Pharmacia Biotechnology, Piscataway, NJ). After fixing cells with methanol, high molecular mass [^{3}H]-radioactivity was precipitated using 5% trichloroacetic acid at 4˚C for 16 h. After two washes with PBS, [^{3}H]-radioactivity was solubilized in 0.2 N NaOH and 0.1% SDS and determined using a liquid scintillation counter (Beckman Instruments, Fullerton, CA). Each experiment was performed in triplicate.

Invasion assay. The invasive chemotactic motility of HUVECs was assayed using a Transwell chamber with 6.5-mm diameter polycarbonate filters (8-μm pore size, Corning Costar, NY, USA). Briefly, the lower surface of the filter was coated with 10 μg of gelatin, and the upper surface of the filter was coated with 10 μg of Matrigel (BD Biosciences, Bedford, MA). The indicated concentration of PHBP and bFGF (25 ng/ml) prepared in 600 μl of EBM with 1% FBS and 0.1% BSA were placed in the lower side. HUVECs were trypsinized and suspended at a final concentration of $5x10^5$ cells/ml in EBM containing 1% FBS. Cell suspension (100 μl) was loaded into each of the upper wells. The chamber was incubated at 37˚C for 16-20 h. Cells were fixed in methanol and stained with hematoxylin and eosin. Non-migrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and invasive cells were quantified by counting the

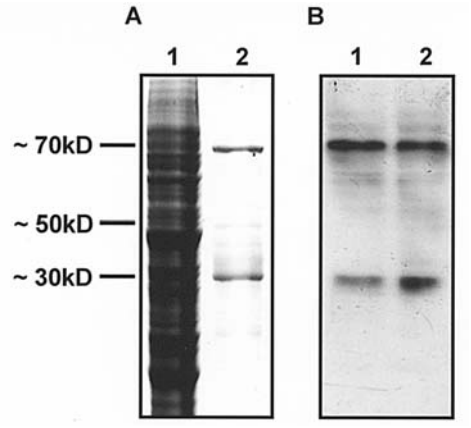

Figure 1. Expression and purification of mouse recombinant PHBP from human embryonic kidney 293 cells. Mouse PHBP cDNA was in-frame cloned into pcDNA3.1/V5-His-TOPO, transfected into HEK293 cells, and selected by G418. Conditioned medium was harvested from HEK293 cells and analyzed by SDS-PAGE on 10% gel under reducing conditions. (A) The gel was stained with Coomassie blue. (B) Western blotting was performed with anti-V5 antibody. Lane 1, total protein of conditioned medium; lane 2, sample eluted from conditioned medium using the Ni-NTA magnetic agarose beads.

cells that migrated to the lower side of the filter using optical microscopy.

Tube formation assay. Matrigel (10 mg/ml) was loaded in a 24-well plate and polymerized for 30 min at 37˚C. HUVECs incubated in EBM with 1% FBS for 6 h were harvested after trypsin treatment, resuspended in EBM with 1% FBS, plated onto a layer of Matrigel at a density of $4x10^5$ cells/well, and followed by the addition of PHBP. Matrigel cultures were incubated at 37˚C. After 12 or 24 h, the cultures were photographed (x40 magnification). The area covered by the tube network was photographed.

Western blotting. Cells were lysed with ice-cold lysis buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5% NP-40 and 1% Triton X-100] containing 1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Sigma). Conditioned medium from HEK293 were also obtained with 0.2 mM PMSF and protease inhibitor cocktail. Protein was quantitated using the BCA method with bovine serum albumin (BSA) as standard, and separated by SDS-PAGE, and electroblotted to a polyvinyldifluoride (PVDF) membrane. The membrane was blocked for 2 h at room temperature in PBS-T and 5% non-fat dry milk, and then incubated with primary antibody for 2 h at room temperature. After washing with PBS-Tween-20, the membrane was incubated with secondary antibody for 1 h and visualized using ECL reagents according to the manufacturer's instructions.

Construction of the truncated plasmids of PHBP. To examine whether the kringle domain of PHBP is responsible for the anti-angiogenic effects of PHBP, 3'-end truncated mutants of PHBP cDNA were constructed with pcDNA3.1/V5-His-TOPO. One removed only the serine protease domain (termed ΔS) and the other removed both the kringle and serine protease domain (termed ΔKS). ΔS and ΔKS were produced by PCR from wild-type PHBP expression vector, pcDNA3.1/PHBP-V5-His wild-type. The following primers were used: for ΔS, forward ATGTTTGTCAGGATGTTGTGTTT and reverse ACGCT TGACTGCGTGTTCAGCT; for ΔKS, forward ATGTTTGT CAGGATGTTGTGTTT and reverse GTCGTCCGGACCTA TTTCACAG.

Data analysis and statistics. All data shown are as means ± SD or as percentage of control, and representative data from one of three replicate experiments are shown. Statistical comparisons between groups were performed using the Student's t-test. Data were considered statistically significant at $P<0.05$.

Results

Expression and purification of recombinant mouse PHBP protein from human embryonic kidney 293 cells. To examine the effects of PHBP on the angiogenesis of endothelial cells induced by bFGF, full-length mouse PHBP cDNA was constructed. The cDNA encoding mouse PHBP protein was in-frame cloned into pcDNA3.1/V5-His-TOPO, and then the vector was transfected into HEK293 cells. The expression of mouse PHBP protein of the conditioned medium was verified by Coomassie blue staining and Western blot analysis (Fig. 1A and B, lane 1, respectively). Purified recombinant mouse PHBP using Ni-NTA magnetic agarose beads was detected in lane 2. Single-chain PHBP (70 kDa) and light chain of active two-chain form PHBP (30 kDa) were observed by Western blot analysis with anti-V5 antibody under reducing conditions (Fig. 1B).

PHBP inhibits angiogenesis on chick CAM. The anti-angiogenic activity of PHBP was investigated in the chick *in vivo* CAM assay, which is a useful model to investigate the effect of compounds on basal angiogenesis (23). Purified PHBP, added to the CAM, significantly inhibited the formation of blood vessels in a dose-dependent manner (Fig. 2). At the

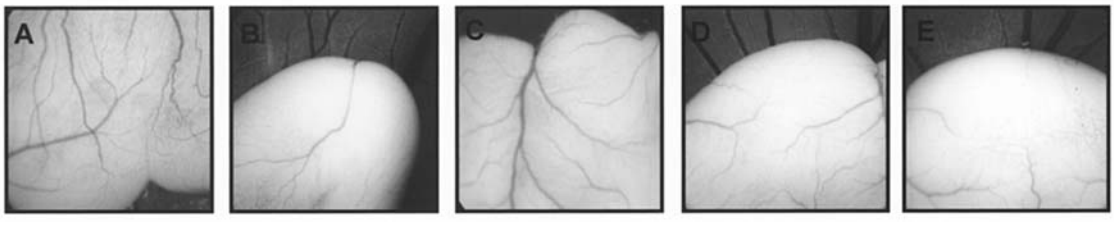

Figure 2. PHBP inhibits angiogenesis on chick CAM. Thermanox coverslips containing variable amounts of PHBP were applied to the CAMs of 4.5-day-old chick embryos. After 48 h of incubation, 10% fat emulsion (Intralipose) was injected into the CAM for observation of the inhibition zone of angiogenesis under a microscope. (A) Control; (B) retinoic acid (1 μg/disc), positive control; (C) 0.1 μg/disc of PHBP; (D) 1 μg/disc of PHBP; (E) 10 μg/disc of PHBP.

Table I. Anti-angiogenic effect of PHBP on the chick CAM.

Compounds	Dose (μg/egg)	Eggs showing angiogenesis inhibition	Total eggs tested	% of inhibition (mean ± SD)
Control[a]	-	2	12	16.7±4.7
Retinoic acid[a]	1	9	13	69.2±12.8[b]
PHBP	0.1	6	22	27.3±5.7[b]
	1	11	21	52.4±13.1[b]
	10	13	14	92.9±23.2[b]

Anti-angiogenic responses were scored as positive when the PHBP-treated CAM showed an avascular zone similar to retinoic acid-treated CAM, which had a [a]few vessels compared with control, and were calculated by the percentage of positive eggs. [b]P<0.05 versus control.

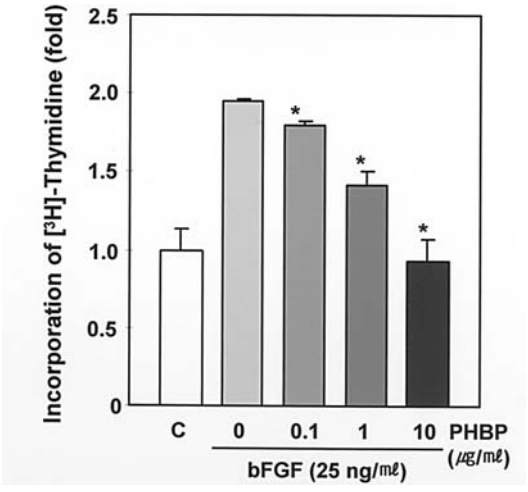

Figure 3. PHBP inhibits bFGF-induced HUVEC DNA synthesis. The indicated concentrations of PHBP were added to HUVECs stimulated with bFGF (25 ng/ml) and incubated for 24 h. [^{3}H]-thymidine was presented during the last 4 h of incubation. Data represent the means ± SD of triplicate, and similar results were obtained in at least three different experiments. C, untreated control cells. *P<0.05 versus bFGF alone.

maximum concentration (10 μg/disc), PHBP inhibited angiogenesis at 92.9% of the treated embryos (Table I). This finding indicates that PHBP prevents angiogenesis *in vivo*, affecting spontaneous angiogenesis of the embryo. The normal structure of the other components of the CAM, such as the chorionic and allantoic epithelia, was not affected, suggesting a selective effect of PHBP on the vascular structures.

PHBP inhibits bFGF-induced HUVEC proliferation. The process of angiogenesis requires endothelial cell proliferation, extracellular matrix degradation and invasion through the underlying basement membrane and interstitial matrix and, finally, spatial organization to form a network of new vessels (24). Therefore, we investigated whether PHBP could affect endothelial cell functions relevant to angiogenesis *in vitro*. Since kringle structure affected endothelial cell growth on angiogenesis (8), we verified the anti-proliferative effect of

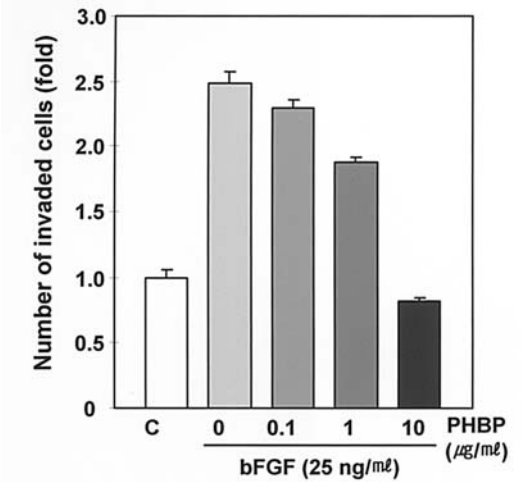

Figure 4. PHBP inhibits bFGF-induced HUVEC invasion. The indicated amounts of PHBP were placed in the lower side of Transwell with bFGF (25 ng/ml) in EBM containing 1% FBS. Data represent the means ± SD of duplicate, and similar results were obtained in at least three different experiments. C, untreated control cells. *P<0.05 versus bFGF alone.

PHBP on HUVECs using [^{3}H]-thymidine incorporation assay. PHBP inhibited the DNA synthesis of HUVECs in a dose-dependent manner (Fig. 3). PHBP at 10 μg/ml inhibited entirely bFGF-induced HUVEC proliferation. The proliferation of HUVECs induced by vascular endothelial growth factor (VEGF) was also inhibited in a dose-dependent manner (data not shown).

PHBP inhibits bFGF-induced HUVEC invasion. To examine whether PHBP regulates endothelial cell invasion, the ability of HUVECs to penetrate the reconstituted basement membrane, Matrigel, was assessed. PHBP decreased the invasion of HUVECs in a dose-dependent manner (Fig. 4). The result was obtained when PHBP had been added to the lower side of the Transwell, but a dose-dependent addition of PHBP to the upper side of the Transwell did not inhibit the invasion of HUVECs (data not shown). These data suggest that interaction of PHBP and bFGF is important to inhibit the invasion of HUVECs.

PHBP inhibits bFGF-induced HUVEC tube formation. To determine whether PHBP has an inhibitory effect on the tube formation of endothelial cells, the effect of PHBP on the alignment of endothelial cells in capillary-like structures was evaluated (Fig. 5). HUVECs were plated onto a thick layer of Matrigel where they rapidly aligned forming tubes. These tubes became stronger and more robust with longer networks as time of treatment with bFGF progressed. In contrast, the presence of PHBP caused a concentration-dependent inhibition of bFGF-induced tube formation. PHBP at 10 μg/ml inhibited the network formation of HUVECs, resulting in less extensive, broken, foreshortened, and much thinner vessels at many sites.

The kringle domain of PHBP is essential to inhibit bFGF-induced HUVEC tube formation. To determine which domain of PHBP is responsible for the inhibitory effect on angiogenesis, mutant forms of PHBP, ΔS (serine protease domain-deleted form of PHBP) and ΔKS (both kringle and serine

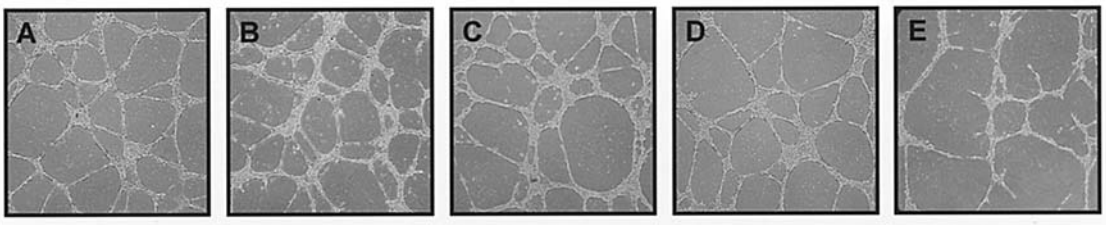

Figure 5. PHBP inhibits bFGF-induced tube formation of HUVECs. HUVECs were treated with various concentrations of PHBP and plated onto a thick layer of pre-coated Matrigel (10 mg/ml) in a 24-well plate. The morphological changes of HUVECs in the surface of Matrigel were investigated. Images were taken 12 h after plating (x40 magnification). (A) Control; (B) bFGF (25 ng/ml); (C) bFGF + 0.1 μg/ml of PHBP; (D) bFGF + 1 μg/ml of PHBP; (E) bFGF + 10 μg/ml of PHBP.

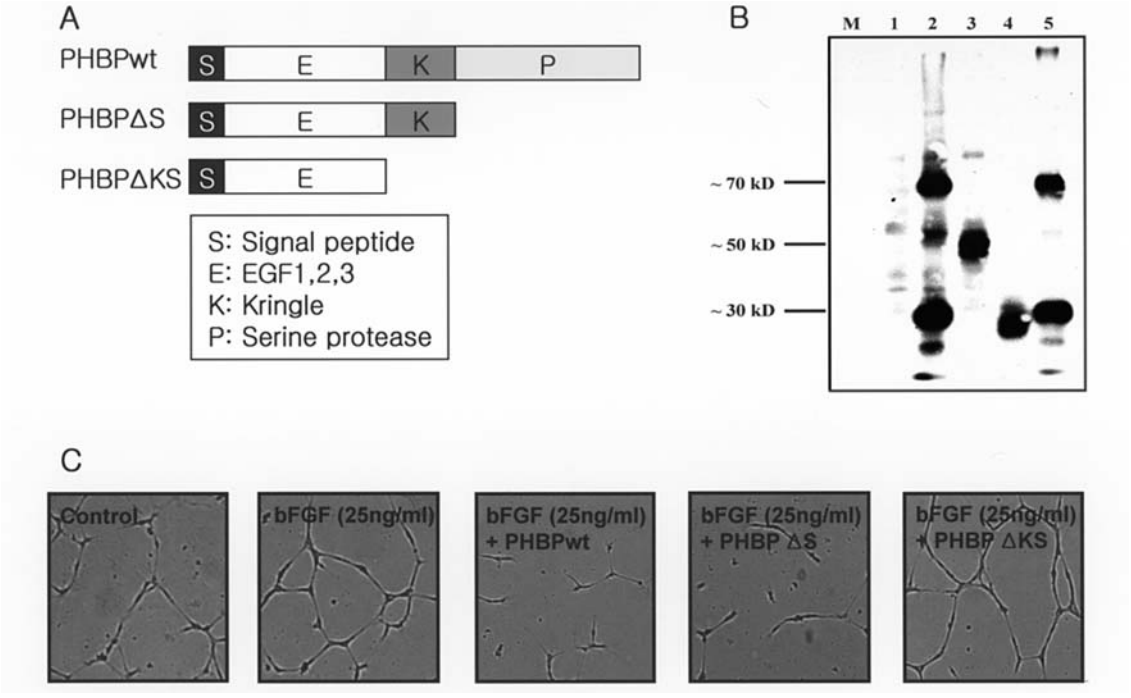

Figure 6. The kringle domain of PHBP is essential to inhibit bFGF-induced tube formation of HUVECs. (A) PHBPΔS was a deleted serine protease domain from C-terminus of PHBP. PHBPΔKS was a deleted kringle domain and a serine protease domain from C-terminus of PHBP, only three EGF domains of PHBP remained. (B) Conditioned medium was harvested from HEK293 cells and analyzed by SDS-PAGE on 10% gel under reducing conditions. Western blotting was performed with anti-V5 antibody. Lane 1, untransfected; lane 2, wild-type PHBP; lane 3, ΔS; lane 4, ΔKS; lane 5, stable transfectants of wild-type PHBP. (C) The kringle domain of PHBP is essential to inhibit bFGF-induced tube formation of HUVECs. Images were taken 24 h after plating (x40 magnification). Similar results were obtained in two different experiments.

protease domain-deleted form) were constructed (Fig. 6A). The expression of ΔS (~50 kDa) and ΔKS (~30 kDa) on the conditioned media of HEK293 cells were verified by Western blot analysis (Fig. 6B). The overexpression of ΔS, the serine protease domain-deleted form of PHBP, effectively inhibited bFGF-induced tube formation on HUVECs and its anti-angiogenic effect was similar to that shown by the treatment of wild-type PHBP. However, the ΔKS-treated HUVECs failed to form a blood vessel network (Fig. 6C).

Discussion

In order to identify novel angiogenesis inhibitors, we searched proteins containing a kringle domain by using a gene screening method with structural basis and focused on PHBP.

PHBP is known as a regulator in hemostasis, which is a tissue factor-independent activator of factor VII promoting early steps of the coagulation pathway (12), and contributes to the fibrinolytic pathway by activating plasminogen activator (25). In addition to the roles described above, it is possible that PHBP has another function on endothelial cells, since PHBP is abundantly present in human plasma and contains a kringle domain within its structure. Therefore, this study was conducted to gain insights into the cellular effects of PHBP on vascular endothelial cells.

As a function independent of the role of PHBP in the hemostatic system, we clearly elucidated the possible role(s) of PHBP in angiogenesis by performing *in vivo* and *in vitro* angiogenesis assays. We first tested the effect of PHBP on angiogenesis in chick embryo and then observed that PHBP significantly inhibits the development of capillary networks in CAM (Fig. 2).

We next observed the effects of PHBP on angiogenesis in each step by using *in vitro* angiogenesis assays, since the

angiogenic process is a tightly regulated phenomenon that includes sequential steps. PHBP markedly inhibited bFGF-induced proliferation (Fig. 3) and invasion (Fig. 4) of HUVECs in a dose-dependent manner. It is noted that both bFGF-induced proliferation and invasion of HUVECs were completely inhibited by treatment of 10 μg/ml PHBP.

To determine the effect of PHBP on HUVEC differentiation, we conducted a tube formation assay. In the presence of bFGF, HUVECs, placed on the Matrigel, established a tube-like structure that seemed to be indicative of an early stage of angiogenic development, whereas HUVECs in the presence of PHBP hardly formed capillary-like networks (Fig. 5).

In addition, we also found that anti-angiogenic activities of PHBP do not seem to depend on the specificity of growth factor. PHBP efficiently prevents the proliferation, invasion and tube formation of HUVECs induced by bFGF or VEGF in a dose-dependent manner (Fig. 3; unpublished data). This is consistent with the most recent study that PHBP inhibits bFGF/EGF-induced proliferation of HUVECs (18).

Collectively, our findings that PHBP blocked endothelial cell proliferation, invasion and tube formation *in vitro* suggest that PHBP acts at a multi-step process of the angiogenic cascade, and might explain its potent anti-angiogenic activity *in vivo*. Especially, the angiostatic activities of PHBP were validated at a concentration of 10 μg/ml, which is close to the amount of PHBP under physiological conditions (12 μg/ml). These results provide evidence that the physiological role of PHBP is not restricted to hemostasis but also affects angiogenesis.

PHBP contains a kringle domain which shares some homology with the kringles of plasminogen. Kringle domains are independent structural and functional folding units found in several proteins involved in blood coagulation and fibrinolysis (8). A series of studies show that hemostasis and angiogenesis are increasingly interrelated. During vessel repair, the regulation of angiogenesis is mediated by proteins secreted by the hemostatic system, which include cryptic fragments from the coagulation cascade and fibrinolytic system.

Kringles 1-4 (angiostatin) or kringle 5 of plasminogen are known for their anti-angiogenic activity by selectively inhibiting endothelial cell growth (26). In addition, kringles derived from other molecules such as kringle 2 of prothrombin and kringles of human hepatocyte growth factor were also found to be inhibitors of endothelial cell proliferation (27,28).

Therefore, we speculate that the anti-angiogenic properties of PHBP may be mediated by kringle structure and investigated whether the kringle domain is essential to the anti-angiogenic activity of PHBP. We performed tube formation assay with two kinds of mutants, ΔS (serine protease domain-deleted form of PHBP) and ΔKS (both kringle and serine protease domain-deleted form) (Fig. 6). We found that the serine protease domain of PHBP was not necessary to the anti-angiogenic effect. However, the serine protease and kringle domain-truncated mutant was deprived of the anti-angiogenic ability of PHBP. This result indicates that the kringle domain of PHBP is essential to the anti-angiogenic activity of PHBP.

We demonstrate the structural and functional relationships of the kringle domain of PHBP in the regulation of angiogenesis. However, the molecular mechanisms of the anti-angiogenic action of PHBP still need to be defined. Although PHBP exists at a high concentration in human plasma, PHBP might not exert the inhibitory effect of angiogenesis under normal physiological conditions. Consequently, it might play an important role in maintaining the angiostatic condition. In physiological and pathological conditions, such as wound healing or tumor progression, PHBP may be activated and then inhibit angiogenesis through inhibiting endothelial functions and interfering with angiogenic signalings.

Our results indicate that PHBP potently inhibits multiple angiogenic processes *in vitro* and *in vivo*, and that these anti-angiogenic actions of PHBP are mediated by the kringle structure, a conserved architecture that specifically inhibits angiogenesis.

Acknowledgements

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