Pituitary Tumor-Transforming Gene (PTTG) Induces both Vascular Endothelial Growth Factor (VEGF) and Basic Fibroblast Growth Factor (bFGF)

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Angiogenesis is tightly regulated by a variety of angiogenic activators and inhibitors. Disruption of the balanced angiogenesis leads to the progress of diseases such as cancer, rheumatoid arthritis, and diabetic blindness. Even though a number of proteins involved in angiogenesis have been identified so far, more protein factors remain to be identified due to complexity of the process. Here I report that pituitary tumor-transforming gene (PTTG) induces migration and tube formation of human umbilical vein endothelial cells (HUVECs). High levels of both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are detected in conditioned medium obtained from cells transfected with PTTG expression plasmid. Taken together, these results suggest that PTTG is an angiogenic factor that induces production of both VEGF and bFGF.

Key Words : Pituitary tumor-transforming gene, Vascular endothelial growth factor, Basic fibroblast growth factor, Angiogenesis

Introduction

Angiogenesis, the generation of new blood vessels from pre-existing vessels, is a multi-step mechanism that encompasses activation, migration, and proliferation of endothelial cells. Angiogenesis in physiological processes is critical for development, reproduction, and wound healing. Angiogenesis is strictly regulated by a balance of many angiogenic and anti-angiogenic factors. When the balance is disrupted, it leads to the progress of diseases such as cancer, rheumatoid arthritis, and various blood vessel-related disorders.[1-3] Like normal tissues, tumor tissues need angiogenesis for tumor growth that requires the supply of oxygen and other essential nutrients. In addition, newly formed blood vessels are a route for metastasis. Therefore, treatment of cancer with anti-angiogenic drugs will block cancer cell growth and metastasis. Due to explosive research in angiogenesis, a variety of angiogenic and anti-angiogenic factors have been identified.[4] So far, more than 20 angiogenic factors and 300 anti-angiogenic factors have been identified. Of them, VEGF and basic fibroblast growth factor (bFGF) have prominent activities in angiogenesis.[5]

Since angiogenesis is controlled by multiple regulation mechanisms, more endogenous anigogenic regulators might be involved in the process. In an effort to search for endogenous angiogenic factors, I found that pituitary tumor-transforming gene (PTTG) is involved in angiogenesis. PTTG is expressed at low levels in most normal human tissues but is highly expressed in malignant human cell lines and in pituitary tumors.[6] PTTG has been shown to regulate bFGF secretion.[6,7] However, it is not clear whether PTTG is also capable of inducing another angiogenic factor, VEGF. It has been reported that PTTG might induce angiogenesis via VEGF.[8] In contrast, it was also reported that PTTG expression may not be involved in production of VEGF.[9]

In the present study, I show that conditioned medium (CM) derived from human embryonic kidney (HEK) transfectants overexpressing wild-type human PTTG (PTTG-CM) induces the migration and tube formation of human umbilical vein endothelial cells (HUVECs). PTTG-CM contains high levels of VEGF and bFGF as determined by ELSIA assays, which suggests that PTTG is related to production of both VEGF and bFGF. Taken together, these results suggest that PTTG may be a physiological regulator of angiogenesis with the ability to induce both VEGF and bFGF.

Results and Discussion

Conditioned Medium from PTTG-overexpressing HEK 293 Cells Stimulates Endothelial Cell Migration. Angiogenesis process is composed of various steps in which many endogenous proteins are involved for regulation. Since endothelial cell migration through extracellular matrix is one of the essential steps for neovascularization, I first investigated the effects of several candidate genes on endothelial cell migration. For these experiments, I transfected HEK 293 cells with plasmids bearing genes of interest and Zeocin marker for selection. After selection of cells stably expressing specific proteins in Zeocin-containing medium and confirmation of protein expression by immunoblotting (Fig. 1A), CM was collected from Zeocin-resistant cells for HUVEC migration assays. In a chemotaxis chamber, HUVECs were treated with CM from stably transfected cells for 2 h and HUVECs that had been migrated through membrane pores were counted under microscope. I used VEGF (20 ng/mL) as a positive control. Among those, CM from PTTG-overexpressing HEK 293 cells (PTTG-CM) showed significantly induced migratory effect, compared to that of control-CM (CM obtained from empty plasmid-

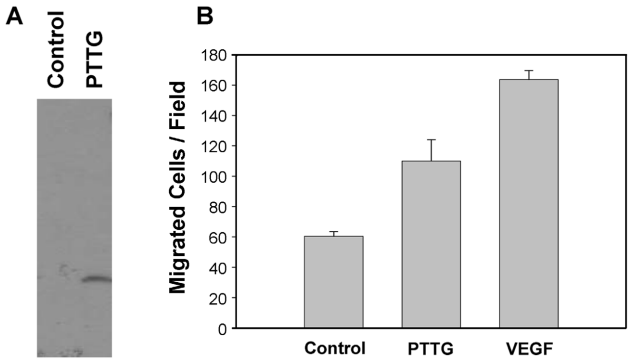

Figure 1. PTTG expression in stably transfected HEK 293 cells and HUVEC migration assays. (A). Stably transfected cells were lysed in a buffer solution, followed by centrifugation at 13,000 rpm for 30 min. Cell lysates were run in SDS-PAGE and transferred to nitrocellulose membrane. The blocked membrane was then incubated with an anti-HA antibody, followed by an appropriate secondary antibody conjugated to horseradish peroxidase. The immunoreactive bands were visualized using an ECL system. PTTG ≅40 KDa. (B). PTTG-CM stimulates endothelial cell migration. Migration assays with human umbilical vein endothelial cells (HUVECs) were carried out in 48-well microchemotaxis chambers. HUVECs were incubated with control-CM or PTTG-CM for 2 h. After fixation and staining, the number of migrated cells was determined by counting two regions of each well under a microscope. VEGF (20 ng/mL) was used as a positive control.

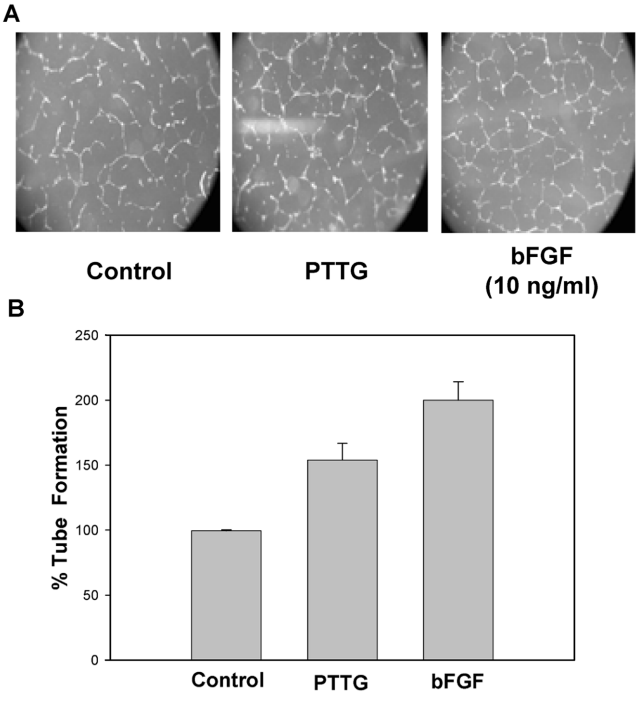

Figure 2. CM from PTTG-overexpressing cells promote tube formation of endothelial cells. (A). HUVECs were collected and seeded on Matrigel-coated plates at a density of 2.4×10^4 cells/well and then incubated with control-CM or PTTG-CM. As a positive control, bFGF (10 ng/mL) was used. After 18 h, fields from each sample were photographed. Representative images of tube formation activity are shown. (B). Total tube areas were analyzed and quantitated by the Scion Image program. The control tube areas were defined as 100% tube formation, and the percent increase in tube formation as compared with control was calculated for each sample.

transfected cells) (Fig. 1B). This result indicates that CM from cells stably overexpressing PTTG (PTTG-CM) has the biological activity that can induce endothelial cell migration.

PTTG induces HUVEC tube formation. Next, to determine whether PTTG functions for the HUVEC tube formation that is a major process of angiogenesis,[10] I examined the ability of PTTG to promote the formation of capillary-like structures of HUVECs on Matrigel. PTTG-CM caused an increase in capillary-like structures, compared to control-CM (Fig. 2). Therefore, these data indicate that PTTG has the ability of inducing tube formation of endothelial cells.

Effect of PTTG on VEGF and bFGF Secretion into Conditioned Medium. I next measured the protein levels of VEGF and bFGF in CM to investigate whether VEGF and bFGF productions are involved in PTTG-stimulated HUVEC tube formation since they are ubiquitous and potent activators of angiogenesis. Using ELISA assay kit, I found that PTTG-overexpressing cells released significantly more VEGF and bFGF than control cells (Fig. 3). These results suggest that the increased levels of secreted VEGF and bFGF may be associated with PTTG-induced angiogenic activity involved in migration and tube formation, at least in part.

In conclusion, the results of this study suggest that both angiogenic factors, VEGF and bFGF, may be effecters for PTTG-mediated angiogenesis. This observation provides

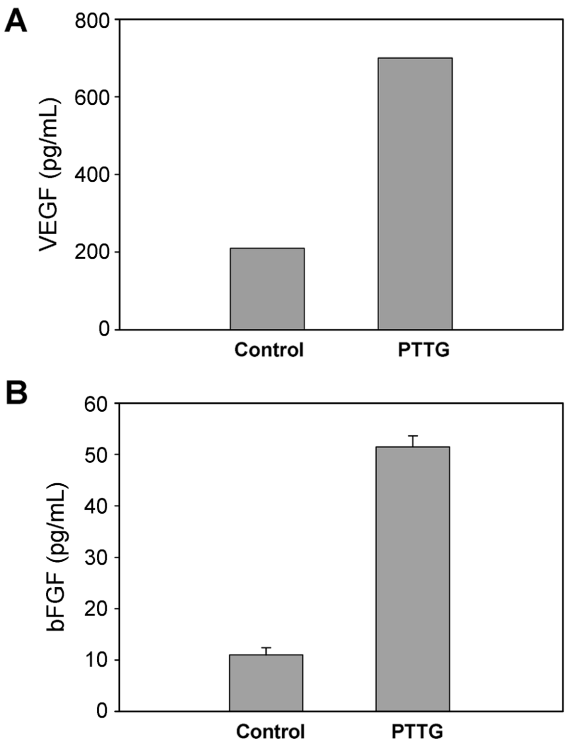

Figure 3. Effect of PTTG on VEGF and bFGF secretion into conditioned medium. Control or PTTG-expressing stable cells were incubated in serum-free M199 for 20 h, and aliquots of the CM were collected. The levels of VEGF and bFGF in CM were measured by ELISA as described in Material and Methods. This is a representative of three independent experiments.

evidence that PTTG might be a candidate for developing anti-cancer agent in tumor growth and angiogenesis.

Experimental Section

Cell Culture. Human embryonic kidney (HEK) 293 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with 0.375% sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS) in a humidified 5% CO_2 incubator at 37 °C. Primary human umbilical vein endothelial cells (HUVECs) were obtained from POSTECH and maintained on gelatin-coated dishes in M199 medium supplemented with 20% FBS, 5 units/mL of heparin, 3 ng/mL basic fibroblast growth factor (bFGF), and penicillin/streptomycin. The HUVECs used in this study were from passages 5 to 8.

Plasmid Construct. The N-terminal hemagglutinin (HA)-tagged human PTTG gene for expression in mammalian cells was constructed by polymerase chain reaction, followed by cloning into the pcDNA3.1/Zeo plasmid.

Transfection and Selection of Transfected Cells. HEK 293 cells were transfected by lipofectamine (Invitrogen) with 1 μg each of pcDNA3.1/Zeo-HA- PTTG or pcDNA3.1/Zeo-empty vector as a control. After 48 h, transfected cells were selected for two weeks in DMEM containing 10% FBS and Zeocin (200 μg/mL). After 15 days of selection, resistant cells arising in dishes were propagated in low level (150 μg/mL) of Zeocin. The overexpressed PTTG in stably transfected cells was confirmed by immunoblotting.

Immunoblotting. Cells were lysed in a buffer solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 μg/mL aprotinin for 30 min at 4 °C, followed by centrifugation at 13,000 rpm for 30 min. Cell lysates were run in SDS-PAGE and transferred to nitrocellulose membrane. The blocked membrane was then incubated with an anti-HA antibody (Santa Cruz Biotechnology, Inc.), followed by an appropriate secondary antibody conjugated to horseradish peroxidase. The immunoreactive bands were visualized using an ECL system.

Preparation of Conditioned Medium. Confluent cells were washed and grown in serum free M199 medium. After 20 h of incubation, conditioned medium (CM) was collected and centrifuged at 500 × g for 10 min and then at 800 × g for 20 min to remove debris. The resultant CM was immediately used for migration and tube formation assays or stored at −70 °C until use.

HUVEC Migration Assays. Endothelial cell migration assays were carried out in a 48-well microchemotaxis chamber (Neuro Probe Inc., Cabin John, MD) as described.[11] Polycarbonate membrane with 12-mm pore was coated with 0.1% gelatin. HUVECs were resuspended in 0.1% BSA/M199. The bottom chamber was loaded with 30,000 cells and the membrane was laid over the cells. Invertation and incubation of the chamber were carried out in sequence. After 2 h incubation, upper wells were loaded with serum free M199 medium and CM samples. The chamber was reincubated for 2 h and membrane filter was fixed and stained using Diff-Quick (Baxter Healthcare Corp.) The number of cell that migrated through the filter was counted under a microscope.

Capillary-like Tube Formation Assays. Tube formation assays were performed with commercial GFR Matrigel (BD bioscience). Forty-eight-well plates were coated with 150 μL GFR matrgel (10 mg/mL) and incubated at 37 °C for 30 min to promote polymerization. Briefly, total 0.3 mL mixtures of HUVECs (24,000 cells/well) and CM samples were added to each well coated with GFR matrigel. After 18 h incubation, fields from each sample were photographed, and total tube areas were analyzed by the Scion Image program. The control tube areas were defined as 100% tube formation, and the percent increase in tube formation as compared with control was calculated for each sample.

Determination of the VEGF and bFGF Expression Levels. To determine the VEGF and bFGF protein expression levels, I obtained CM from control or stable cells. The amounts of VEGF and bFGF proteins in the CM from the cells were determined using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Quantification of protein expression levels were analyzed in equal number of cells.

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