Anti-angiogenic Activity of the Recombinant Kringle Domain of Urokinase and Its Specific Entry into Endothelial Cells*

Kwang Sei Kim‡§, Yong-Kil Hong§§, Young Ae Joe§§, Yoon Lee‡, Jooyoung Shin‡, Hyo-Eun Park‡, Il-Ha Lee, Soo-Young Lee, Dong-Ku Kang§§, Soo-Ik Chang§§§, and Soo Il Chung §§

From the ‡Cancer Research Institute, Catholic Research Institutes of Medical Sciences, and the §Department of Natural Sciences, College of Medicine, The Catholic University of Korea, Seoul 137-701, the ||Department of Biochemistry, College of Natural Science, Biotechnology Research Institute, Chungbuk National University, Cheongju 361-763, the ¶¶Cancer Metastasis Research Center, Yonsei University College of Medicine, Seoul 120-752, and the §§§Mokp Biotechnology Research Institute, Yongin 449-910, Korea.

Urokinase plasminogen activator (uPA) belongs to a family of proteins that contains kringle domain and plays an important role in inflammation, tissue remodeling, angiogenesis, and tumor metastasis by pericellular plasminogen activation. Kringle domains of plasminogen have been shown to demonstrate anti-angiogenic and anti-tumor activities. Here, we report our investigation of the kringle domain of uPA for anti-angiogenic activity and a possible cellular mechanism of action. The recombinant kringle domain of uPA (Asp45-Lys135) (UK1) inhibited endothelial cell proliferation stimulated by basic fibroblast growth factor, vascular endothelial growth factor (VEGF), or epidermal growth factor. It also inhibited migration of endothelial cells induced by VEGF or uPA, and in vivo angiogenesis on the chick chorioallantoic membrane. It did not block plasminogen activation by activated uPA in clot lysis and chromogenic substrate assays. Neither binding of UK1 to immobilized uPA receptor nor competitive inhibition of uPA binding were confirmed by real-time interaction analysis. However, internalization of UK1 followed by translocation from cytosol to nucleus was determined to be specific to endothelial cells. It also elicited a transient increase of Ca2+ flux of more than 2-fold within 2 min of exposure in an endothelial cell-specific manner. These results suggest that the kringle domain of uPA exhibits anti-angiogenic activity and that its anti-angiogenic activity may occur through a different mechanism from inhibition of uPA-uPA receptor interaction or uPA proteolytic activity and may be associated with endothelial-cell specific internalization not mediated by the uPA receptor.

Urokinase-type plasminogen activator (uPA or urokinase) has been implicated in inflammation, angiogenesis, tissue remodeling, and the progression and metastasis of numerous solid tumors (1–6). uPA is a multidomain protein composed of a carboxyl-terminal protease domain and an amino-terminal fragment, which can be further subdivided into a growth factor-like domain (aa 4–43) and a kringle domain (aa 45–135) (7). Urokinase is a highly specific serine protease that converts plasminogen into plasmin. It also binds to a specific glycolipid-anchored receptor (uPA receptor) with high affinity, primarily via its growth factor-like domain, to mediate proteolysis on the cell surface.

Angiogenesis is a complex, multistep process that occurs during normal physiology such as wound healing, pregnancy, and development, as well as under pathological conditions such as diabetic retinopathy and tumorigenesis (8–11). During angiogenesis, endothelial cells need to divide, migrate, invade the extracellular matrix, and form capillary structures from pre-existing blood vessels (12). This complex process implies the presence of multiple controls, which can be turned on and off within a short period. There is an increasing body of evidence showing that inhibition of angiogenesis can lead to the suppression of tumor growth and metastasis (13–16).

Among the family of endogenous angiogenesis inhibitors, angiostatin has been demonstrated to be derived from plasminogen (14). Angiostatin was first isolated from serum and urine of tumor-bearing animals and found to consist of the first four kringles of plasminogen (14). In vitro, it specifically inhibits endothelial cell proliferation but not proliferation of other cell types, including tumor cells (14, 17). In vivo, angiostatin suppresses neovascularization and suppresses tumor growth in animals without toxicity (14, 18). However, its cellular mechanism of action remains unclear.

In general, a kringle is composed of 78–80 amino acids interconnected by a triple disulfide-linked loop. The triple disulfide bonds are strictly conserved between kringle 1 (19). The identity of amino acid sequence between plasminogen kringles

* This work was supported in part by grants from the Korea Health 21 R&D Project, Ministry of Health & Welfare (02-PJ1-PG10-20802-0007), the National Projects of the Korea Ministry of Science and Technology (98-G-08-03-A-26), and the Korea Science and Engineering Foundation through the Cancer Metastasis Research Center at Yonsei University and the Vascular System Research Center at Kangwon National University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this work.

¶ To whom correspondence should be addressed: Cancer Research Institute, Catholic Research Institutes of Medical Science, The Catholic University of Korea, Banpo-dong 505, Seocho-ku, Seoul 137-701, Korea. Tel.: 82-2-590-2404; Fax: 82-2-532-0575; E-mail: youngjoe@catholic.ac.kr.

Received for publication, December 4, 2002, and in revised form, January 3, 2003. Published, JBC Papers in Press, January 15, 2003, DOI 10.1074/jbc.M212358200
averages around 50%. Interestingly, each kringle of angiotatin has been demonstrated to display differential effects on endothelial cell proliferation and migration (17, 20). Besides angiotatin, another plasminogen kringle, PK5, has also been shown to be a potent inhibitor of endothelial cell proliferation and migration in vitro (21, 22). Kringle domains derived from other molecules such as kringle 2 of prothrombin and kringle 4 of human hepatocyte growth factor were also found to be inhibitors of endothelial cell proliferation (23-25), whereas contradictory results have been reported in the anti-endothelial cell activity of human plasminogen kringle 5 (27).

Because angiotatin is a cryptic fragment derived from plasminogen, we were interested to learn whether the urokinase kringle domain exhibits anti-angiogenic activity, although urokinase itself promotes angiogenesis. The urokinase domain also showed a low degree of sequence identity with the plasminogen kringle domains. In this work we examined the effects of the recombinant kringle domain of urokinase in anti-angiogenic activity. To explore the mechanism of action for the anti-angiogenic activity, we tested whether the kringle affects uPA proteolytic activity or interacts with uPA receptor. We also demonstrated that the internalization of the recombinant kringle domain is specific to endothelial cells, which might explain in part why it has endothelial cell-specific activity. In addition, we report an elevation in intracellular Ca²⁺ concentration elicited by the kringle domain of urokinase, specifically in endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**Recombinant human single chain-uPA (sc-uPA) expressed in Chinese hamster ovary cells was obtained from Mogen Biotech Research Institute (Kyungki-do, Korea). Two chain-uPA (tc-uPA) was kindly provided by Green Cross Co. (Kyungki-do, Korea). Angiotatin, containing kringles 1–4, was prepared by digestion of human plasminogen with porcine elastase as published previously (28) and was further purified by size-exclusion chromatography on a Bio-Gel P-200 column. Recombinant human uPA receptor (uPAR), anti-human uPAR polyclonal antibody (AF907), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) were purchased from R&D Systems (Minneapolis, MN). The reagents used for cell culture, M199, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and trypsin solution were purchased from Invitrogen (Grand Island, NY). Endothelial cell growth medium (EGM) and endothelial cell basal medium (EBM)-2 were purchased from Clonetics (San Diego, CA). Angiogenin was purified from cow’s milk as described previously (29). Control mouse isotype IgG was provided kindly by Dr. Jeong-Im Sin (The Catholic University of Daegu, Korea). Polyclonal antiserum were raised against the human recombinant urokinase kringle domain by injecting BALB/c mice three times with 100 µg of the recombinant protein. The IgG fraction was then purified from this antisera by protein A chromatography.

**Cell Culture—**Human umbilical vein endothelial (HUVE) cells were isolated from fresh cords by an adaptation of the method described by Jaffe et al. (30) and maintained in M199 medium containing 20% FBS, 30 µg/ml endothelial cell growth supplements (Sigma, St. Louis, MO), 90 µg/ml heparin, 25 mM Hepes, 2.2 g/liter sodium bicarbonate, 2 mM L-glutamine, and 1% antibiotics. Cells at passage 3, 4, or 5 were used for the experiments. Bovine capillary endothelial (BCE) cells were obtained from Dr. T. H. Lee (23) and maintained in DMEM containing 10% FBS, 1% antibiotics, and 3 ng/ml bFGF. NIH 3T3 and HEK-293 cells were cultured in DMEM supplemented with 10% FBS. Cultures were kept at 37 °C in a humidified atmosphere of 10% CO₂ in air for BCE cells and in 5% CO₂ in air for HUVE, NIH 3T3, and HEK-293 cells. In this study, the BCE cells were maintained in a humidified atmosphere of 10% CO₂ in air for BCE cells and in 5% CO₂ in air for HUVE, NIH 3T3, and HEK-293 cells.

**Construction of the Expression Vectors—**Total RNA was isolated from HUVE cells by using a Qiagen total RNA isolation kit, and cDNAs were synthesized using an oligo(dT) primer and the First Strand cDNA synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany). cDNA encoding the amino acids spanning from Ser1 to Lys135 of human uPA was amplified by PCR with Pfui polymerase (Stratagene, La Jolla, CA) and was used as a forward primer (5'-CAGAATTCCTAGGGCCAGCAATGAACCTCATCACGTTCC-3') and reverse primer (5'-GCCTGGAGGTTCCTACATCGGCCAGCT-3'). The amplified cDNA was introduced into a blunt vector T-Blue according to the manuactu of Perfectly T-Blues blunt cloning kit (Novagen, Madison, WI). The nucleotide sequence of constructed was confirmed by manual sequencing using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham Biosciences, Uppsala, Sweden). For the removal of the NcoI site, PCR-directed mutagenesis was carried out without any change of amino acid sequence by two rounds of PCR reactions. In the first reaction, either forward primer C (5'-ATGCGGCCGCGCCGCCTG-3') and reverse primer B, or forward primer A and primer D (5'-CAGCCAGGGCCG-GCCCATAGTGTACGTCGCTCC-3') were used as a primer set, and the above cDNA was used as a template (the change from G to A is indicated by boldface type). In the second round, primers A and B were used as a primer set, and two PCR products from the first rounds were used as templates. Two PCR products were subjected to sonication for 15 min (8,000 × g). Refolding and purification of UK1 were carried out as follows. The cell pellet was suspended in a lysis buffer (20 mM Tris-Cl, pH 8.0, 50 mM NaCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 20 µg/ml RNase A, 50 µg/ml DNase I) and subjected to sonication for 15 min. The soluble and insoluble fractions were obtained as described for 30 min at 25 °C. The soluble fraction was dissolved in buffer A (20 mM Tris-Cl, pH 7.9, 0.5 mM NaCl, 6 µl urea). After centrifugation, the supernatant solution was applied to a ProBond™ resin His tag affinity column (Invitrogen, Carlsbad, CA). The column was washed with buffer A containing 20 mM imidazole, and protein was eluted with buffer A containing 1 M imidazole. The eluate was stepwise diluted with 0.1 M Tris-Cl, 0.15 M NaCl, pH 8.0, followed by addition of reduced (1 mM) and oxidized (0.1 M) glutathione and incubation at room temperature overnight. The solution was concentrated by ultrafiltration and dialyzed against 20 mM sodium acetate buffer (pH 5.5) and distilled water. The dialyzed solution was lyophilized, dissolved in 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 buffer, and subjected to size exclusion chromatography on a Superose 6 column equilibrated in the same buffer. The endotoxin content of purified UK1 used for cell assays did not exceed 0.6 endotoxin unit/mg of protein.

**Analysis of Protein—**Amino acid sequence was determined with an Applied Biosystems, Model Procise 491, protein sequencing system at the Korea Basic Science Institute (Seoul, Korea). Matrix-assisted laser desorption ionization mass spectrometry was performed using a Voyager Biospectrometry workstation with a linear mass analyzer (PerSeptive Biosystems, Framingham, MA) at the Korea Basic Science Institute (Seoul, Korea). Endothelial Cell Proliferation Assay—Endothelial cell proliferation assay was performed as previously described (14, 32). 12,500 cells/well were seeded in a 24-well plate and were cultured in DMEM containing 10% FBS, 1% antibiotics, and 2 ng/ml bFGF. The cells were cultured in 2% CO₂ for 24 h. The media was replaced with 0.25 ml of DMEM, 5% FBS, 1% antibiotics, and the test sample applied. After 30 min of incubation, 0.25 ml of DMEM, 5% FBS, 1% antibiotics, 2 ng/ml bFGF was added to each well. After 72 h, cells were dispersed in trypsin and counted by a trypan blue exclusion method.

**In vivo Activity—**HUVE cells, [3H]thymidine incorporation was performed as follows: 2 × 10⁴ cells/well were plated into gelatinized 24-well culture plates and were incubated in M199, 10% FBS, 90 µg/ml heparin, 1% antibiotics for 24 h. The media was replaced with 0.25 ml of DMEM, 5% FBS, 1% antibiotics, and the test sample applied. After 30 min of incubation, 0.25 ml of DMEM, 5% FBS, 1% antibiotics, 2 ng/ml bFGF was added to each well. After 72 h, cells were dispersed in trypsin and counted by a trypan blue exclusion method.
and dissolved in 0.25 M NaOH, 1% SDS. Radioactivity was determined using a liquid scintillation counter. Each sample was assayed in duplicate.

**Endothelial Cell Migration Assay**—To evaluate migration, a modified Boyden chamber-based assay (33) was performed. Transwell polycarbonate membrane (8-µm pore size) (Costar, Cambridge, MA) was coated with 0.1% gelatin. After HUVE cells were trypsinized and suspended in M199 medium (4 × 10^5 cells/ml), 100 µl was added to each well in the upper chamber. The filter was placed over a bottom chamber containing M199 medium, 2 mg/ml VEGF, 0.1% bovine serum albumin. For testing inhibitory activity, HUVE cells were preincubated with inhibitors in M199 medium in the cell culture incubator for 30 min before being added to the upper chamber, and the inhibitor was added to the chamber. The assemblies were incubated at 37 °C with 5% CO2, to allow cells to migrate through the gelatin-coated polycarbonate filter. The filter membrane was removed from the chamber, fixed with methanol, and stained with hematoxylin Gill No. 2 (Sigma) and eosin. Non-migrated cells on the upper surface of the filter were removed by scraping with a cotton swab. The total number of migrated cells with nuclei per well was counted using a digital photographic system. Each experiment was performed in triplicate.

The migration of HUVE cells by uPA was determined using a “scratch” wound assay (34–36). Briefly, HUVE cells were plated in six-well culture plates at 5 × 10^4 cells/cm² and cultured in EGM. When the cells are confluent, they were washed with prewarmed PBS and then incubated in EBM-2 supplemented with 1% FBS for 24 h. The cell monolayer was scraped with a cell scraper to create a cell-free zone. After washing three times with EBM-2, HUVE cells were treated with uPA in the absence or in the presence of UK1 (10–500 ng/ml) in EBM-2 containing 1% FBS. HUVE cell migration was quantified by measuring the width of the cell-free zone (distance between the edges of the injured monolayer) after 24 h under a Leica DM IRB real-time inverted microscope. Each sample was assayed in duplicate, and the assays were repeated twice.

**Chorioallantoic Membrane Assay**—To determine antiangiogenic activity in vivo, a CAM assay was performed as previously described (37, 38). Fertilized eggs (Pulmuone, Kyungki-do, Korea) were incubated at 37 °C with 50%–60% relative humidity. At day 2, a portion of the shell was removed and a window was made on day 3. At day 4.5 of incubation, test samples were loaded on a quarter size Thermanox coverslip (Nunc, Roskilde, Denmark) were applied on the CAM of individual embryos. After another 2-day incubation, a 20% fat emulsion was injected into the CAM for observation of the inhibition zone of angiogenesis. The vascular networks in the CAMs were blindly scored for the presence or absence of an avascular zone larger than 5 mm in diameter.

**Plasminogen Activation Assay**—Human fibrinogen was prepared by a modification of the method of Mosesson et al. (39). The ability of the fibrinogen to clot was higher than 95% when thrombin was added. Fibrin clot plates were prepared from fibrinogen (3 mg/ml) containing human fibrinogen and 0.2 mg/ml in buffer NaCl, 50 mM Tris–HCl, pH 7.6) by adding of human thrombin (Sigma, 0.4 unit/ml) and incubating for 1 h at 37 °C. Aliquots of PBS containing tc-uPA (10 pmol in 10 µl of PBS) in the presence or absence of an excess molar concentration (50–100-fold) of UK1 were overlaid on the fibrin clot plate. After incubation at 37 °C for 1 h, the diameter of the clear zone was photographed and measured.

Ten microliters of the substrate (N-tosyl-Gly-Pro-Lys-p-nitroanilide, Sigma) solutions (3 mM) was mixed with 0.2 ml of HEPES buffer, pH 8.0 (0.1 mM, 0.16 mM NaCl, 0.1% bovine serum albumin), and 10 µl of plasminogen (1 mg/ml), and prewarmed to 25 °C. Aliquots of PBS containing tc-uPA (1.5 pmol in 10 µl of PBS) in the presence or absence of and the addition of 50 µl of plasminogen (1 mg/ml). The reaction was stopped by the addition of 50 µl of acetic acid. The digestion of the substrate was determined by measuring increased absorbance of free p-nitroaniline at 405 nm.

**Binding Assay by a BIAcore Biosensor**—An assay of UK1 and uPA binding was performed by real-time interaction analysis using a BIAcore 2000™ apparatus (BIAcore AB, Uppsala, Sweden). All experiments were performed at 20 °C using PBS buffer containing 0.005% (v/v) surfactant P20 (running buffer). Recombinant uPA (20 µg/ml in 10 mM sodium acetate buffer, pH 5.0) was immobilized on a CM-5 type sensor chip of a 5000-gate sensor chip with the amine coupling kit (BIAcore AB). The samples of UK1, sc-uPA, and tc-uPA were diluted in the running buffer before injection. For general binding assay, each sample was injected over the human uPA at a flow rate of 10 µl/min. The kinetics of binding was measured at a buffer flow of 30 µl/min, including an injection phase of 3 min followed by dissociation for 5 min. Sensor chips were regenerated at the end of each run by the injection of 0.05 M acetic acid, 0.25 M NaCl. The BIAevaluation™ software was used for the subtraction of bulk effects (flow cell 1) and a blind curve obtained with buffer alone to compensate for drift. The sensorgram was analyzed by non-linear curve fitting using the BIAevaluation™ software, version 3.0.2, assuming single-site association model with a single-site dissociation model. If the kinetic analysis failed, the kinetic model was ascertained by evaluating the resulting fitted curves, using the residual plot function of the software (requiring a random point scattering for the fit to be accepted) as well as calculation of χ² (requiring a χ² value below 10 in both the association and dissociation phase data). The results were expressed as dissociation constant (K_D) and the dissociation rate constant (k_d). Calculations and the kinetic analysis were performed with the software (BIAevaluation) assuming single-site association (K_D) and single-site dissociation (k_d) as well as calculation of χ² (requiring a χ² value below 10 in both the association and dissociation phase data). The results were expressed as dissociation constant (K_D) and the dissociation rate constant (k_d). Calculations and the kinetic analysis were performed with the software (BIAevaluation) assuming single-site association (K_D) and single-site dissociation (k_d) for deriving the equilibrium dissociation constant (K_D = k_d/k_a).

**Cellular Localization of Fluorescein-labeled UK1 in HUVE Cells**—Fluorescein-labeled UK1 was prepared by reaction of the fluorescein succinimidy1 ester (Pan Vera, Madison, WI) according to the manufacturer’s protocol. HUVE cells were trypsinized, seeded at 5 × 10⁴ cells/cm² on a 18 × 18-mm cover glass placed in six-well culture plates, and cultured in EGM for 24 h. After washing with prewarmed PBS, HUVE cells were cultured in EBM-2 supplemented with 1% FBS for 24 h. The cells were washed three times with prewarmed (37 °C) EBM-2. A wash cover glass was mounted in a modified chamber positioned on the platform of an inverted confocal microscope (Carl Zeiss). To test the effect of the inhibitor, the cells in prewarmed (37 °C) EBM-2 with fluorescein-labeled UK1 (1 µg/ml), exchange of the 0.5-ml chamber volume was achieved with manual volume replacement by pipetting from the above. After 30 min, the chamber was washed with prewarmed (37 °C) EBM-2, and then fluorescence was observed with a confocal laser scanning microscope (Carl Zeiss LSM510). The cells were incubated with 10 µg/ml Hoechst 33342 for 20 min, washed with EBM-2, and then fluorescence was observed with a fluorescence microscope (Carl Zeiss).

**Effect of UK1 on the Concentrations of Ca²⁺ in the HUVE, NIH 3T3, and HEK-293 Cells**—Cells were trypsinized and seeded, at 5 × 10⁴ cells/cm², on a 18 × 18-mm cover glass placed in six-well plates. HUVE cells were cultured in EGM for 24 h, and NIH 3T3 and HEK-293 cells were cultured in DMEM supplemented with 10% FBS for 24 h. After washing with prewarmed PBS, HUVE cells were cultured in EBM-2 supplemented with 1% FBS for 24 h, and NIH 3T3 and HEK-293 cells were cultured in DMEM for 24 h. HUVE cells were washed three times with prewarmed (37 °C) EBM-2, and NIH 3T3 and HEK-293 cells were washed with prewarmed (37 °C) DMEM. The cells on the cover glass were incubated in the medium containing 4.4 µM Fluo-3AM (Molecular Probes, Eugene, OR) for 40–60 min. After washing the cells three times with prewarmed (37 °C) EBM-2 or DMEM, the washed cover glass was mounted in a modified chamber positioned on the platform of a Zeiss inverted laser confocal microscope. The cells in prewarmed (37 °C) EBM-2 or DMEM were incubated with UK1 (5 µg/ml) as described in the above, and the fluo-3AM fluorescence images were measured at a given time by a confocal laser scanning microscope (Carl Zeiss LSM510).

**RESULTS**

**Production and Purification of UK1**—Because the kringle domain (UK1) of urokinase has no glycosylation site (7), we expressed it in bacteria. The recombinant plasmid constructed for UK1 expression contains 6 histidine residues at the carboxyl-terminal end. Most of the expressed protein appeared in inclusion bodies (IB) as an insoluble form. The solubilized IB protein was purified using a Ni^2+–chelate column, refolded in vitro, and purified to homogeneity by size exclusion chromatography as described under “Experimental Procedures” (Fig. 1A). The yield of purified protein was about 20% of the isolated IB. The purified UK1 migrated on SDS-PAGE as a single band of about 13–14 kDa under reducing conditions. Under non-reducing conditions, it migrated a little faster, corresponding well with the expected molecular weight (Fig. 1B). Mass spectrometric analysis indicated a molecular mass of 11,595 Da, in agreement with the calculated mass of 11,595 Da (6 Da have been subtracted due to the three disulfide bonds formed). From the amino-terminal sequenc-
matched exactly the predicted sequence of the recombinant protein.

Dose-dependent Inhibition of Endothelial Cell Proliferation by UK1—Purified UK1 was assessed for its inhibitory activity on BCE cell growth stimulated by bFGF (1 ng/ml). UK1 exerted a potent growth inhibitory effect on bFGF-stimulated BCE cells in a dose-dependent manner (Fig. 2A). The concentration of UK1 required to reach 50% inhibition (ED₅₀) was about 80 nM, similar to that for angiostatin. On the other hand, uPA itself did not affect the proliferation of BCE cells. BCE cells exposed to UK1 for 30 min and washed prior to bFGF addition resulted in a dose-dependent inhibition of cell proliferation similar to the cell proliferation inhibition pattern observed without UK1 removal (data not shown). The proliferation of HUVE cells induced by bFGF, VEGF, or EGF was also inhibited in a dose-dependent manner (Fig. 2B). The anti-endothelial proliferative activity of UK1 does not seem to depend on a specific growth factor. UK1 produced as a soluble protein from a Pichia expression system also showed a similar potency in inhibition of HUVE cell proliferation induced by several growth factors. However, the addition of high levels of UK1 (up to 640 nM) did not affect the proliferation of NIH 3T3 fibroblast and HEK-293 epithelial cells under the same culture conditions (data not shown).

As shown in Fig. 2C, the inhibitory activity of UK1 on endothelial cell proliferation was blocked by an anti-UK1 antibody, whereas neither anti-uPAR antibody nor control IgG blocked its inhibitory activity. We confirmed the interaction of UK1 and the anti-UK1 antibody by an immunoprecipitation method.

Inhibition of VEGF- or uPA-induced Endothelial Cell Migration by UK1—Because the migration response of the endothelial cells is better with VEGF than bFGF (40), VEGF-induced migration was employed to evaluate anti-migratory activity of UK1. VEGF (2 ng/ml) was used in the low chamber to stimulate the migration of HUVE cells subcultured to passage P3–P4. In this assay, angiostatin inhibited the VEGF-induced HUVE cell migration in a dose-dependent manner (data not shown). As shown in Fig. 3, UK1 also displayed a dose-dependent inhibition in the concentration range of 10–10,000 ng/ml, with an IC₅₀ value of about 1 nM. In the absence of VEGF, we could not detect any effect of UK1 (at up to 1,000 ng/ml concentration) on migration of HUVE cells.

Because uPA itself is able to stimulate endothelial cell motility at nanomolar concentrations (41), we tested whether UK1 can block the migration of endothelial cells induced by uPA. As in the modified Boyden chamber method using VEGF, UK1 blocked the migration of HUVE cells induced by uPA in a dose-dependent manner in wound migration assay (Fig. 3B).

Anti-angiogenic Activity of UK1 in the Chick Embryo—To evaluate the possible ability of UK1 to inhibit in vivo angiogenesis, we used the chick chorioallantoic membrane assay (37, 38). Over a concentration range of 1–100 μg/embryo, UK1 inhibited angiogenesis in a dose-dependent manner (Fig. 4). With repeated doses of 20 μg/embryo, we confirmed that the inhibition is reproducible. No toxicity was observed in the embryos tested.

**Fig. 1.** Purification and SDS-PAGE analysis of UK1. A, samples obtained from the purification steps were subjected to electrophoresis on a 14% SDS-polyacrylamide-gel in Tris-glycine buffer under reducing conditions; the gel was stained with Coomassie Blue. Lane 1, total protein of E. coli; lane 2, soluble fraction of E. coli lysate; lane 3, insoluble fraction from E. coli lysate; lane 4, flow-through fraction from the His tag Ni²⁺ affinity column; lane 5, sample eluted from the His tag affinity column; lane 6, purified UK1 after the size exclusion column. B, purified UK1 was analyzed under reducing condition (lane 1) and non-reducing condition (lane 2).

**Fig. 2.** Inhibition of endothelial cell proliferation by UK1. A, inhibition of BCE cell proliferation induced by bFGF. Anti-proliferative activity was assayed with BCE cells in the presence of 1 ng/ml bFGF for 72 h as described under “Experimental Procedures.” The effects of UK1 (closed circles) were compared with those of angioatin (open circles) and sc-uPA (closed triangles). Values, given as percentages of inhibition, represent the mean of three determinations (±S.E.). B, inhibition of HUVE cell proliferation by UK1. The cells were cultured with 3 ng/ml bFGF, 10 ng/ml VEGF, or 10 ng/ml EGF with or without various amounts of UK1 for 18 h, followed by incubation with [³H]thymidine as described under “Experimental Procedures.” C represents a condition of no growth factor and no inhibitor. Each value represents the mean ± S.E. C, effects of anti-UK1 and anti-uPAR antibodies on UK1 inhibitory activity. The cells were cultured in the absence or presence of 3 ng/ml bFGF with or without 320 nM UK1 preincubated with control antibody (100 μg/ml) or anti-UK1 antibody (100 μg/ml). In the case of anti-uPAR antibody, it was added directly to the cells 45 min before addition of UK1. A [³H]thymidine incorporation assay was followed. Results are expressed as the percentages of DNA synthesis in the absence of inhibitor UK1 (as 100%).
The Effects of UK1 on Plasminogen Activation by uPA—Movement of endothelial cells involves proteolysis of the extracellular matrix. Two families of matrix degradation proteases, the plasminogen activator and the matrix proteinases, can, in concert, degrade most extracellular proteins (2). Therefore, we examined whether UK1 can inhibit plasminogen activation by activated uPA. In a fibrin clot lysis analysis, the presence of a 50- or 100-fold molar excess of UK1 did not change any activity of tc-uPA for plasminogen activation (Fig. 5).

In a more sensitive chromogenic substrate assay, UK1 also did not inhibit the activity of tc-uPA at all. The velocities (nanomoles of p-nitroaniline released/min) of amidolytic activity of plasmin generated by tc-uPA were almost the same in the absence of UK1 (1.34 ± 0.05), and the presence of a 50-fold molar excess of UK1 (1.37 ± 0.19) or a 100-fold molar excess of UK1 (1.35 ± 0.02). The concentration of tc-uPA (7.5 nM) used in this assay was chosen in the concentration range (0.5–10 nM) showing the linear relationship between enzyme concentration and final cleaved chromogenic substrate amount in the presence of excess amount of plasminogen and its chromogenic substrate. From these results, we concluded that UK1 does not inhibit the proteolytic activity of uPA and, thereby, does not affect plasminogen activation by activated uPA.

Analysis of the Interaction of UK1 with Immobilized uPAR—Although the amino-terminal growth factor-like domain of urokinase (aa 4–43) is known as a major binding site for uPAR (42), the possible presence of more than one kind of interaction was supported by some reports (43, 44). In an effort to re-evaluate any interaction of UK1 with uPAR, an in vitro study was carried out using BIAcore. Recombinant human uPAR was immobilized directly to a carboxylated dextran matrix chip through amine groups on uPAR, and then UK1, or uPA was injected over the uPAR surface. Representative sensorgrams are shown in Fig. 6 (A and B). sc-uPA was observed to bind with high affinity (k_D = 1.66 ± 0.2 × 10^-7 M^-1 s^-1, k_a = 3.05 ± 0.3 × 10^-3 s^-1, and K_D = 1.84 nM) as expected, whereas UK1 did not show any binding under identical conditions over a concentration range of 15.6–250 nM. Furthermore, it also did not block the binding of uPA to the immobilized receptor when added at various molar ratios of uPA and UK1 (1:1, 1:2, or 1:4) (Fig. 6C).

Thus, the observed UK1 inhibitory effects on in vitro endothelial proliferation, migration, and in vivo angiogenesis do not seem to be related to the uPA receptor.

Endothelial Cell-specific Internalization and Nuclear Localization of UK1—To investigate the pattern of interaction of UK1 with endothelial cells, HUVE cells were incubated with fluorescein-labeled UK1 at 37 °C for 30 min. When cell-associated UK1 was detected by a confocal microscope, UK1 appeared to have been taken up and distributed in both nucleus and cytosol of these cells (Fig. 7A). Therefore, we addressed
whether internalization of UK1 is specific to endothelial cells. After treatment of NIH 3T3 and HEK-293 cells with the fluorescein-labeled UK1, and we observed its movement for 30 min. Internalization of UK1 was not detected in NIH 3T3 and HEK-293 cells during a period of 30 min, in contrast to HUVE cells (Fig. 7, B and C, respectively).

UK1-induced Transient Elevation of Ca²⁺ Concentration in Endothelial Cells—Because intercellular Ca²⁺ concentration ([Ca²⁺]), regulates cell migration (45, 46), progression through the cell cycle (47, 48), and apoptosis (49, 50), we examined if any change in [Ca²⁺] occurs upon exposure to UK1. HUVE cells were first equilibrated with 4.4 μM fluo 3-AM for 40–60 min, washed with serum-free medium, and then incubated with UK1 (5 μg/ml). As shown in Fig. 8A, UK1 elicited two- to 3-fold transient increase of Ca²⁺ levels in cytosol and nucleus within 2 min and returned to the base line in 3 min. The second lower transient mount of Ca²⁺ increase was followed after 5 min. The transient Ca²⁺ flux by UK1 was not observed in non-endothelial cells tested, i.e. NIH 3T3 and HEK-293 cells (Fig. 8B).

DISCUSSION

uPA has one kringle domain, which shares some homology with the kringles of plasminogen, but is distinct in not being capable of binding lysine residue. The kringle 1–4 (angiostatin), or kringle 5 of plasminogen, are known for their anti-angiogenic activity by selectively inhibiting endothelial cell growth (14, 21). In this study, our data demonstrate the potent anti-angiogenic activity of the kringle domain of urokinase, although urokinase itself is pro-angiogenic (2, 3). Recombinant
UK1 selectively inhibited endothelial cell proliferation and suppressed the migration of VEGF- or uPA-stimulated HUVE cells. Its anti-angiogenic activity was also shown in CAM assays in vivo. More importantly, UK1 was selectively and rapidly internalized and elicited a transient Ca\(^{2+}\) flux in endothelial cells.

Kringle domains are independent structural and functional folding units found in several proteins involved in blood coagulation and fibrinolysis (51). In an effort to elucidate the structure and function of kringle domains, several kringle domains, including plasminogen kringles 2, 3, and 2\(\rightarrow\)3 and tissue-type plasminogen activator kringle 2 have been successfully produced by E. coli expression and a refolding process (52–55). The structures of these recombinant kringles have been proven to be close to native kringle fragments by identification of disulfide bonds, determination of lysine-binding capacity, NMR spectroscopy, or x-ray crystallography. The recombinant kringles 1, 2, 2\(\rightarrow\)3, or 4 of plasminogen obtained from a refolding process were also proven to be functional in angiogenesis inhibition (17). In our work, we were able to produce the recombinant kringle domain of urokinase as a functional form by a similar refolding process, and this protein was as functional as the UK1 produced in a soluble form by Pichia. A successful refolding of the functional recombinant kringle domain of urokinase was also reported by another group, who showed that the monoclonal antibody interacting with the kringle domain of uPA recognizes the intact recombinant kringle domain but not its reduced form (56).

Amino acid sequence alignment of the individual kringle domain of human plasminogen shows that PK1, PK2, PK3, PK4, and PK5 display considerable sequence similarity to each other (46–57\%) (17, 21). UK1 from urokinase, although similar, has somewhat lower sequence identity with the plasminogen kringles: e.g. 36.5\% to PK1, 32.9\% to PK2, 30.4\% to PK3, 37.8\% to PK4, and 40.2\% to PK5 (Fig. 9). One stretch of amino acids, between Val and Leu (underlined in Fig. 9) is distinct in UK1, although well conserved between human and bovine species (79\% identity). Despite these differences, UK1 exhibits anti-angiogenic activity, as demonstrated by our current data.

The individual kringles of plasminogen have been shown to exhibit differential effects on endothelial cell proliferation and migration, probably related to their molecular diversity (17, 20). For example, plasminogen kringles 1, 2, 4, and 5, but not 3, have lysine binding capability (17, 21). Kringle 4 shows a marked functional difference in its anti-endothelial cell proliferative and migratory activities from other kringles. It alone is comparatively inefficient in the suppression of endothelial cell growth (17) but potent in inhibiting endothelial cell migration (20). The lack of lysine binding ability of UK1 does not appear to influence its anti-proliferative activity, which agrees with earlier studies that showed no influence of lysine on plasminogen kringles in anti-proliferation of endothelial cells (17, 32). Interestingly, UK1 is also potent in inhibiting the migration of endothelial cells induced by VEGF. These results taken together strongly support the conclusion that lysine-binding capability has no relationship with anti-endothelial cell activities, even though it seems to be important in the fibrinolysis process. Rather, we presume that other structural characteristics may be involved in anti-endothelial cell activity and specificity.

It is unlikely that the anti-angiogenic activity of UK1 is related to uPAR interaction, because no UK1 binding to immobilized uPA receptor and no competitive inhibition of uPA binding were detected in real-time interaction analysis. Rather than that, the specific internalization of UK1 in endothelial cells may imply that another cellular receptor exists in endothelial cells. Rapid internalization and nuclear localization in endothelial cells may explain in part why exposure of just 30 min can exert an inhibition of BCE cell proliferation. It is not certain at present whether its receptor may be directly involved in the mechanism of action of the kringle domain or whether it may only affect cell-specific entry. Further studies will be done to elucidate this specific receptor and its relevance to anti-endothelial cell activities. Recently, another recombinant kringle domain construct (aa 42–166) of urokinase was reported to have a binding site of low affinity on an unidentified cell surface target for muscle cells and to induce migration of these cells (56). Because recombinant UK1 used here only includes aa 45–135, it may not have the same binding pattern as the one reported in that study. In fact, treatment with UK1 alone did not induce migration of HUVE cells in our hands. In addition, the connecting peptide (aa 136–143) has been reported to inhibit cancer cell invasion and endothelial cell migration as a non-competitive antagonist of the uPA-uPAR interaction (57).

Up to now, the molecular mechanism of action of angiostatin on endothelial cell is not clear. ATP synthase, angiomotin, and integrin \(\alpha_\beta\) were independently found to bind to angiostatin (58–60). Cell cycle arrest and apoptosis resulting from treatment of angiostatin may occur by additional independent mechanisms (61–63). In this study, we have demonstrated that angiogenesis inhibitor UK1 elicited the 2- to 3-fold increase of transient Ca\(^{2+}\) flux into cytosol and nucleus within a 2-min period, specifically in endothelial cells. These results suggest that nuclear localization of UK1 and Ca\(^{2+}\) signaling may be involved in the cellular function of UK1 in the endothelial cells. This notion is also supported by the report of Jiang et al. (64) that potent angiogenesis inhibitors angiostatin and endostatin transiently elevate [Ca\(^{2+}\)]\(i\), in primary cultures of bovine and human endothelial cells. They also reported an interesting result that endostatin pretreatment of endothelial cells for 18 h reduced \(\sim\)70\% the magnitude of acute Ca\(^{2+}\) signals elicited subsequently by VEGF and bFGF. This result may explain, in part, why UK1 and uPA can have opposite effects on endothelial cell migration, although both UK1 and uPA increase [Ca\(^{2+}\)]. It can also be presumed that UK1 may inhibit the alterations of Ca\(^{2+}\)-mobilizing properties, because it was suggested that the alterations of Ca\(^{2+}\) store site-related Ca\(^{2+}\) mobilizations, i.e. Ca\(^{2+}\) sequestration, release, and thapsigargin-evoked Ca\(^{2+}\) entry, may be involved in the cellular mechanisms of endothelial migration (65). Further studies on how
UK1 acts in endothelial cells with respect to Ca$^{2+}$ signaling may provide some insight in more detail into this question.

An increasing number of endogenous angiogenesis inhibitors that become active as a cryptic fragment of an inactive precursor protein have been identified in recent years. For example, angiostatin derived from plasminogen inhibits endothelial cell proliferation and blocks angiogenesis in vivo (14). However, plasminogen is inactive. Similar cases were reported for endothelial cells.

UK1 acts in endothelial cells with respect to Ca$^{2+}$ signaling may provide some insight into this question. At present, we don’t know whether the kringle domain itself circulates in blood or whether it exists in the extracellular matrix of an angiogenic area, even though it has three potential cleavage sites, Lys$^{353}$, Ser$^{47}$, Lys$^{335}$, Lys$^{336}$ and Glu$^{144}$-Lys$^{144}$, near the kringle domain of urokinase (7). It remains to be studied further, together with the mechanism of action of UK1 in its anti-angiogenic activity.

Acknowledgments—We thank Jun-Youne Jeong for technical support for the BIACore assay; Hyun-Joe Ku Kim, and Hyun-Kyung Kim for assistance in antibody generation; and Dr. Edith C. Wolff, NIDCR, National Institutes of Health, Bethesda, MD, for helpful comments on the manuscript.

REFERENCES

1. Blasi, F. (1997) Immunol. Today 18, 415–417

2. Carmeliet, P., and Collen, D. (1998) Kidney Int. 53, 1519–1549

3. Tkachuk, V., Stepanova, V., Little, P. J., and Bobik, A. (1996) Curr. Opin. Oncol. 8, 243–248.

4. Andreasen, P. A., Kjoller, L., Christensen, L., and Duffy, M. (1997) J. Mol. Evol. 45, 531–538

5. Adreasen, P. A., Kjoller, L., Christensen, L., and Duffy, M. (1997) J. Mol. Evol. 45, 531–538

6. Mohanam, S., Go, Y., Sawaya, R., Venkaiah, B., Mohan, P. M., Kouraklis, C. D., Thomas, D. P., and Tuddenham, E. G. D., eds) pp. 575–633. Churchill Livingstone, Singapore

7. Folkmann, J. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 1384–1389

8. O’Reilly, M. S., Holmgren, L., Shing, Y., and Folkman, J. (1994) Cell 79, 315–328

9. O’Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., and Folkman, J. (1997) Cell 88, 277–285

10. Bergers, G., Javaherian, K., Le, K. M., Folkman, J., and Hanahan, D. (1999) Science 286, 808–812

11. Cao, Y., Ji, R. W., Davidson, D., Schaller, J., Marti, D., Sohndel, S., McCance, S. G., O’Reilly, M. S., Linas, M., and Folkman, J. (1996) J. Biol. Chem. 271, 2541–2547

12. Folkmann, J. (1995) New Engl. J. Med. 333, 1757–1763

13. Folkmann, J. (1990) in The Molecular Basis of Cancer (Mendelsohn, J., Howley, P. M., Isak, M., and Liotta, L. A., eds) pp. 206–231. W. B. Saunders, Philadelphia

14. Chang, X., Xu, R., Zhang, Q., Li, T. P., and Gan, B. (2000) Biochem. Biophys. Res. Commun. 276, 178–186

15. Kuba, K., Matsumoto, K., Date, K., Shimizu, H., Tanaka, M., and Nakamura, T. (2000) Cancer Res. 60, 6737–6743

16. Lou, X. J., Kwan, H. W., Priomas, S. D., Yang, Z. J., Lawn, R. M., and Fajardo, L. F. (1998) Exp. Mol. Pathol. 65, 53–63

17. Triere, V. N., and Uckun, F. M. (1999) Biochem. Biophys. Res. Commun. 257, 714–718

18. Kirsch, M., Strasser, J., Allelende, R., Belloe, L., Zhang, J., and Black, P. M. (1998) Cancer Res. 58, 4654–4659

19. Chang, S. I., Pak, S.-H., Seo, S.-H., and Ahn, B.-C. (1996) J. Biol. Chem. 271, 353–358

20. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, R. (1973) J. Cell Biol. 55, 275–276

21. Kim, H.-J., and Park, S. (1999) Bull. Korean Chem. Soc. 20, 370–372

22. Lee, H. S., Kim, H. K., Lee, J. H., You, W. K., Chung, S. I., Chang, S.-L., Park, M. H., Hong, Y. K., Kim, H. K., and Joe, Y. A. (2000) Archiv. Biochem. Biophys. 379, 356–363

23. Yamaguchi, N., Anand-Apte, B., Lee, M., Sasaki, T., Fukai, N., Shapiro, R., Que, L., Lowik, C., Timpl, R., and Olsen, B. R. (1999) EMBO J. 18, 4414–4423

24. Xin, L., Xu, R., Zhang, Q., Li, T. P., and Gan, B. (2000) Biochem. Biophys. Res. Commun. 276, 178–186

25. TASER, Xin, L., Xu, R., Zhang, Q., Li, T. P., and Gan, B. (2000) Biochem. Biophys. Res. Commun. 276, 178–186

26. Kuba, K., Matsumoto, K., Date, K., Shimizu, H., Tanaka, M., and Nakamura, T. (2000) Cancer Res. 60, 6737–6743

27. Lou, X. J., Kwan, H. W., Priomas, S. D., Yang, Z. J., Lawn, R. M., and Fajardo, L. F. (1998) Exp. Mol. Pathol. 65, 53–63