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Differential inhibition of endothelial cell proliferation and migration by urokinase subdomains: amino-terminal fragment and kringle domain

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Abbreviations: bFGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; HUVE, human umbilical vein endothelial; scu-PA, single-chain uPA; u-ATF, amino-terminal fragment of uPA; UK1; kringle domain of uPA; uPA, urokinase plasminogen activator; uPAR, uPA receptor

Abstract

The serine protease urokinase-type plasminogen activator (uPA) is implicated in pericellular proteolysis in a variety of physiological and pathological processes including angiogenesis and tumor metastasis. The kringle domain of uPA (UK1) has proven to be an anti-angiogenic molecule with unknown mechanism and amino terminal fragment of uPA (u-ATF) with additional growth factor-like domain can be used for blocking interaction of uPA and uPA receptor. Here, we compared anti-angiogenic activities of these two molecules *in vitro* and *in vivo*. The recombinant u-ATF from *E. coli* and refolded *in vitro* was found to bind to uPAR with high affinity, whereas *E. coli*-derived UK1 showed no binding by Biacore analysis. In contrast to UK1 having potent inhibitory effect, u-ATF exhibited low inhibitory effect on bovine capillary endothelial cell growth (ED_{50} > 320 nM). Furthermore, u-ATF inhibition of VEGF-induced migration of human umbilical vein endothelial cell was far less sensitive (IC_{50} = 600 nM) than those observed with UK1, and angiogenesis inhibition was marginal in chorioallantoic membrane. These results suggest that kringle domain alone is sufficient for potent anti-angiogenic activity and additional growth factor-like domain diverts this molecule in undergoing different mechanism such as inhibition of uPA/uPAR interaction rather than undergoing distinct anti-angiogenic mechanism driven by kringle domain.

Keywords: angiogenesis inhibitors; angiostatic proteins; cell migration inhibition; endothelial cells; urokinase-type plasminogen activator

Introduction

Urokinase-type plasmingen activator (uPA) and its receptor are important components of cell surface proteolysis used by tumor cells and capillary endothelial cells for basement membrane invasion and implicated in the progression, metastasis, and angiogenesis of numerous tumors (de Vries *et al*,, 1996; Andreasen *et al*., 1997; Reuning *et al*., 1998). uPA is secreted from cells as a single-chain proenzyme that is cleaved by plasmin into its two-chain active form (Petersen *et al*., 1988). Both the single and two-chain forms can bind to u-PA receptor (uPAR). The two chain form consists of A (aa 158) and B (aa 159-411) polypeptide chains linked by disulfide bonds. Single-chain uPA (scu-PA) is a multidomain protein composed of a carboxyl-terminal protease domain, termed low molecular weight scu-PA (LMW-scu-PA) and an amino-terminal fragment (u-ATF), which can be further subdivided into a growth factor-like domain (aa 4-43) and a kringle domain (aa 45-135) (Figure 1A). The general feature of kringle structure is composed of 78-80 amino acids inter-connected by a triple disulfide-linked loop.

The high level expression of uPA and uPA receptor (uPAR) are correlated with a poor prognosis in a num-

ber of malignancies, including breast, gastric, brain and lung cancer (Sappino *et al*., 1987; Yamamoto *et al*., 1994b; Foekens *et al*., 1995; Park *et al*., 1997) and is often localized to the leading, invasive edge of a tumor (Estreicher *et al*., 1990; Yamamoto *et al*., 1994a). Blockage of the interaction of uPA and its receptor has resulted in inhibition of the migration and spreading of malignant tumor cells (Crowley *et al*., 1993; Mohanam *et al*., 2002).

During angiogenesis, endothelial cells need to divide, migrate, invade into the extracellular matrix, and form capillary structures from preexisting blood vessels. The process of cell invasion requires the production of a key active cell surface protease, uPA. Treatment of capillary endothelial cells with angiogenic factors such as basic fibroblast growth factor (bFGF) and VEGF causes up-regulation of uPA (Saksela *et al*., 1987) and uPAR (Mignatti *et al*., 1991; Mandriota *et al*., 1995). Interference with the activities of the uPA system has been demonstrated in some cases inhibition of angiogenesis *in vitro* (Yasunaga *et al*., 1989; Lu *et al*., 1996; Min *et al*., 1996; Fibbi *et al*., 1998), and *in vivo* (Min *et al*., 1996; Li *et al*., 1998).

ATF-based uPAR antagonists are known to have anti-angiogenic function mainly by blocking uPA/uPAR interaction (Lu *et al*., 1996; Li *et al*., 1998). Recently, the recombinant kringle domain of urokinase (UK1) has been shown to present anti-angiogenic activity *in vitro* and *in vivo* by unknown mechanism (Kim *et al*., 2003b). Since u-ATF contains potential two inhibitory domains; a growth factor-like domain where uPAR binding domain is located and a kringle domain, it will be an intriguing question whether u-ATF acts like UK1 on endothelial cells or whether u-ATF elicits more potent anti-angiogenic activity than UK1 due to one additional functional epitope. Therefore, in this study, we compared the anti-angiogenic activities of these two molecules.

Materials and Methods

Materials

Recombinant scu-PA expressed in Chinese hamster ovary cells was obtained from Mogam Biotechnology Research Institute (Kyungki-do, Korea). The reagents used for cell culture, M199, DMEM, FBS and trypsin solution were purchased from Life Technologies (Gaithersbug, MD). VEGF and bFGF were purchased from R&D system (Minneapolis, MN).

Cell culture

Human umbilical vein endothelial (HUVE) cell was isolated from fresh cords by an adaptation of the method described by Jaffe *et al*. (1973), and maintained in M199 medium containing 20% FBS, 30 μg/ml endothelial cell growth supplements (Sigma), 90 μg/ml heparin, 25 mM Hepes, 2.2 g/L sodium bicarbonate, 2 mM L-glutamine and penicillin/ streptomycin antibiotics. Cells at passage 3, 4 or 5 were used for the experiments. Bovine capillary endothelial (BCE) cell was obtained from Dr. TH Lee (Lee *et al*., 1998), and maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin antibiotic, and 3 ng/ml bFGF. Cultures were kept at 37°C in a humidified atmosphere of 10% CO_2 in air for BCE cells and 5% CO_2 in air for HUVE cells.

Expression and purification of u-ATF

A transformant expressing u-ATF was obtained by transformation of *E. coli* BL21 (DE3) with pET-21d/ATF which was constructed previously (Kim *et al*., 2003b). Expression and purification of u-ATF were performed as previously described for UK1 production. A single colony of the transformant was grown in 2.5 liter of LB medium containing 100 mg/ml of ampicillin at 37°C to a A_{600} of about 0.6-0.8. To induce the production of recombinant protein, isopropyl thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1.0 mM. Cells were incubated for an additional 3 h at 37°C and finally harvested by centrifugation for 15 min (8,000 *g*). The cell paste was stored at -70°C. The cell pellet was thawed, suspended in a lysis buffer [20 mM Tris-Cl, pH 8.0, 50 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, RNase A (20 mg/ml), DNase I 50 μg/ml], and subjected to sonication for 15 min. The soluble and insoluble fractions were separated by centrifugation for 30 min at 20,000 *g*. The insoluble fraction was dissolved in a buffer C (20 mM Tris-Cl, pH 7.9, 0.5 M NaCl, 8 M Urea) and diluted to 6 M urea. After centrifugation, the supernatant was applied to a ProBond™ resin His-tag affinity column equilibrated with buffer D (20 mM Tris-Cl, pH 7.9, 0.5 M NaCl, 6 M urea). The column was washed with 20 μM imidazole-containing buffer D, and protein was eluted with 1 M imidazole-containing buffer D. After confirming by SDS-PAGE, the fractions containing the desired protein were combined and stepwise diluted with buffer F (0.1 M Tris-Cl, 0.15 M NaCl, pH 8.0). While diluted, the solution was added with reduced (1 mM) and oxidized (0.1 mM) glutathione and incubated at room temperature overnight. The solution was concentrated by ultrafiltration and dialyzed against sodium acetate buffer (pH 5.5) and distilled water. The dialyzed solution was lyophilized, dissolved in buffer G (20 mM sodium phosphate, 150 mM NaCl, pH 6.8), and applied to a Bio-prep SE 100/17 column equilibrated with the same buffer for the final purification. The endotoxin level of the purified proteins was less than 0.3 U/mg protein.

Binding assay by Biacore biosensor

Binding assays of scu-PA, u-ATF, and UK1 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 (Biacore AB, running buffer). The recombinant uPAR (20 μg/ml in 10 mM sodium acetate buffer, pH 5.0) purchased from R&D system (870-UK/CF) was immobilized on a CM-5 type sensor chip using the reagents and procedures supplied with the Amine Coupling Kit (Biacore AB). The samples of scu-PA, u-ATF, and UK1 were diluted in the running buffer before injection. For general binding assay, each sample was injected over the human uPAR at a flow rate of 10 μl/min. The kinetic of binding were 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 injection of 0.05 M acetic acid, 0.25 M NaCl. All analyses for kinetic data were performed at least in triplicate.

The sensorgrams were analyzed by non-linear curve fitting using the BIAevaluation software, version 3.0.2, assuming single-site association and dissociation models (1:1 binding with drift base line). Optimal sections of each curve was selected, and the applicability of the chosen 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 χ^2 (requiring a χ^2 value below 10 in both the association and dissociation phase for acceptance). Calculated values for the association rate constant (k_a) and the dissociation constant (k_d) were used for deriving the equilibrium dissociation constant ($K_d = k_d/k_a$).

Endothelial cell proliferation assay

BCE proliferation assay was performed as previously described (O'Reilly *et al*., 1994). Cells were washed twice with PBS and trypsinized briefly with 0.05% trypsin solution. Cells were suspended in DMEM, 10% FBS, 1% antibiotics and cell number was counted using a hemocytometer. 12,500 cells/well were plated into gelatinized 24-well culture plates and were incubated (37°C in 10 % CO_2) 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.

Endothelial cell migration assay

To evaluate migration, a modified Boyden chamber based assay was performed (Yamaguchi *et al*., 1999). Transwell polycarbonate membrane with 8 mm pore size (Costar) 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 2 ng/ml $VEGF_{165}$ in 0.1% BSA in M199 medium. For testing the inhibitory activity of recombinant proteins, HUVE cells were pre-incubated with the proteins in M199 medium in the cell culture incubator for 30 min before being added to the upper chamber. The assembled chamber was incubated for 5 h at 37°C with 5% CO_2 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 digital photographic system.

Chorioallantoic membrane (CAM) assay

To determine anti-angiogenic activity *in vivo*, CAM assay was performed as previously described (Kim *et al*., 2003b). Fertilized eggs (Pulmuone, Korea) were incubated at 37°C, and 70% relative humidity. At day 2, a portion of albumin was removed and a window was made on day 3. At day 4.5 of incubation, test samples loaded on a thermanox cover slip (Nunc) of quarter size were applied on the CAM of individual embryos. After another 2 days' incubation, Intralipose^R (KGCC, Korea) was injected into the CAM for observation of inhibition zone of angiogenesis.

Results

Production and purification of u-ATF

The recombinant plasmids constructed for the bacterial expression UK1 and u-ATF contain 6 histidine residues at carboxyl terminal end (Figure 1A). u-ATF was designed to span from Ser(1) to Lys(135), not including 'connecting peptide' (aa 136-158). The expressed protein exists in inclusion bodies as insoluble form. This protein was solublized, purified, and refolded *in vitro*, followed by final purification by size exclusion chromatography to homogeneity. The yield of purified protein is about 20% of the isolated inclusion bodies. The purified u-ATF migrated on SDS-PAGE as a single band at 18 kDa under reducing condition (Figure 1B). Under non-reducing condition, they migrated a little bit faster, corresponding well with the molecular weight expected. The calculated mass for u-ATF is 16,464 Da.

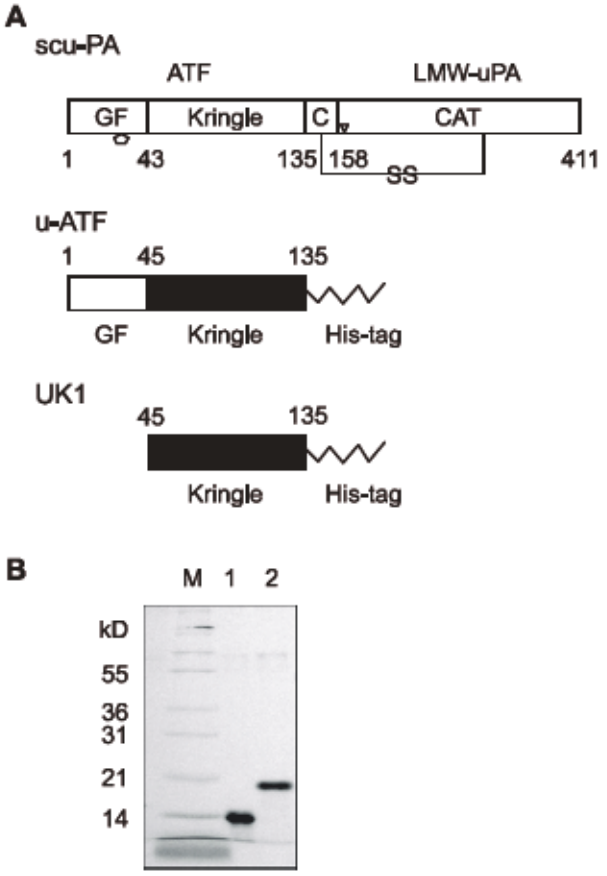

Figure 1. (A) Schematic structure of scu-PA and the recombinant constructs. The recombinant proteins used in this work contain His-tag at the carboxyl-terminus. GF, growth factor-like domain; C, connecting peptide; CAT, catalytic domain; ATF, amino-termianl fragment; LMW-uPA, low molecular weight-uPA; pentagone, fucose at Thr (18); reverse triangle, cleavage site by plasmin (Lys158-Ile159). (B) SDS-PAGE analysis of purified recombinant proteins. Approximately 2 μg of each protein was run on 12% Tris-glycine-SDS gel under reducing condition and stained with Coomassie blue. Lane 1, recombinant kringle domain of uPA (UK1); lane 2, recombinant ATF of uPA (u-ATF).

Analysis of the interaction of UK1, u-ATF, and scu-PA with uPAR by Biacore

ATF domain of uPA has been known to act as an antagonist for uPA receptor (Lu *et al.*, 1996; Li *et al.*, 1998). The interaction of these molecules with uPAR by Biacore assay was carried out in order to verify how refolded proteins, u-ATF and UK1 interact to uPAR with a measurable affinity. Recombinant human uPAR was immobilized directly to a carboxylated dextran matrix chip through amine group on uPAR, and purified UK1, u-ATF, or scu-PA was injected over the uPAR surface. Representative sensorgrams are shown Figure 2A. scu-PA and u-ATF were observed to bind to uPAR with specific affinity as expected. By contrast, UK1 did not show any binding under the identical condition over a concentration range of 15.6 nM to 500 nM, suggesting that kringle domain alone is not able to associate with uPAR (Kim *et al.*, 2003b) (Figure 2B).

Table 1. Kinetic constants measured by real-time interaction analysis Kinetic constants were determined using the Biacore system as described under "Experimental Procedures". ND, no specific binding detected.

Protein samples	$k_a \times 10^{-4}$ $(M^{-1} \cdot s^{-1})^a$	$k_d \times 10^{3}$ $(s^{-1})^a$	K_d $(nM)^b$
scu-PA	166±20	3.05±0.39	1.84±0.00
u-ATF	2.95±0.28	3.56±0.14	122±13
UK1	ND	ND	ND

[a]Rate constants are expressed as the average value found for the three accepted fits in each case±the standard deviation of these determination. [b]The equilibrium dissociation constant, K_d was calculated as the ratio of the rate constants, k_d/k_a.

The kinetic parameters analyzed in another three sets of sensorgrams are summarized in Table I. u-ATF bound to uPAR with high affinity (K_d = 122±13 nM) in a dose-dependent manner. The affinity of u-ATF, which has the K_d value about 66 fold higher than that of the whole molecule, scu-PA, was mainly from the result of a decrease in k_a. In the previous report, ATF exhibits about a 10-50 fold higher ED_{50} in SaOS-2 and U-937 cells than does DFP-uPA in the competitive binding of ^{125}I-ATF (Rabbani *et al.*, 1992). It might suggest that uPA has an additional binding site(s) or modulating domain(s) for uPA/uPAR interaction. These results also indicate u-ATF was properly refolded without significant change in binding affinity for uPAR. In case of krinlge module, bacteria-derived, refolded UK1 and yeast-derived, soluble form both also have proven to elicit almost identical anti-proliferative acivities on endothelial cells in the previous study (Kim *et al.*, 2003a). CD spectra measured for these two proteins also showed almost identical pattern (data not shown).

Effect of u-ATF on endothelial cell proliferation

Purified u-ATF was compared with UK1 for their inhibitory activities on BCE cell growth stimulated by bFGF (1 ng/ml). While UK1 exerted a potent growth inhibitory effect on bFGF-stimulated BCE cells in a dose-dependent manner (Figure 3, ED_{50} of about 80 nM), the inhibitory potency of u-ATF was markedly lower than that of UK1, and its ED_{50} was beyond a concentration range measured ($ED_{50} > 320$ nM). u-ATF displayed a tendency of dose-dependent inhibition, compared to scu-PA which did not affect BCE cell growth at all at a concentration up to 320 nM in the previous study (Kim *et al.*, 2003b).

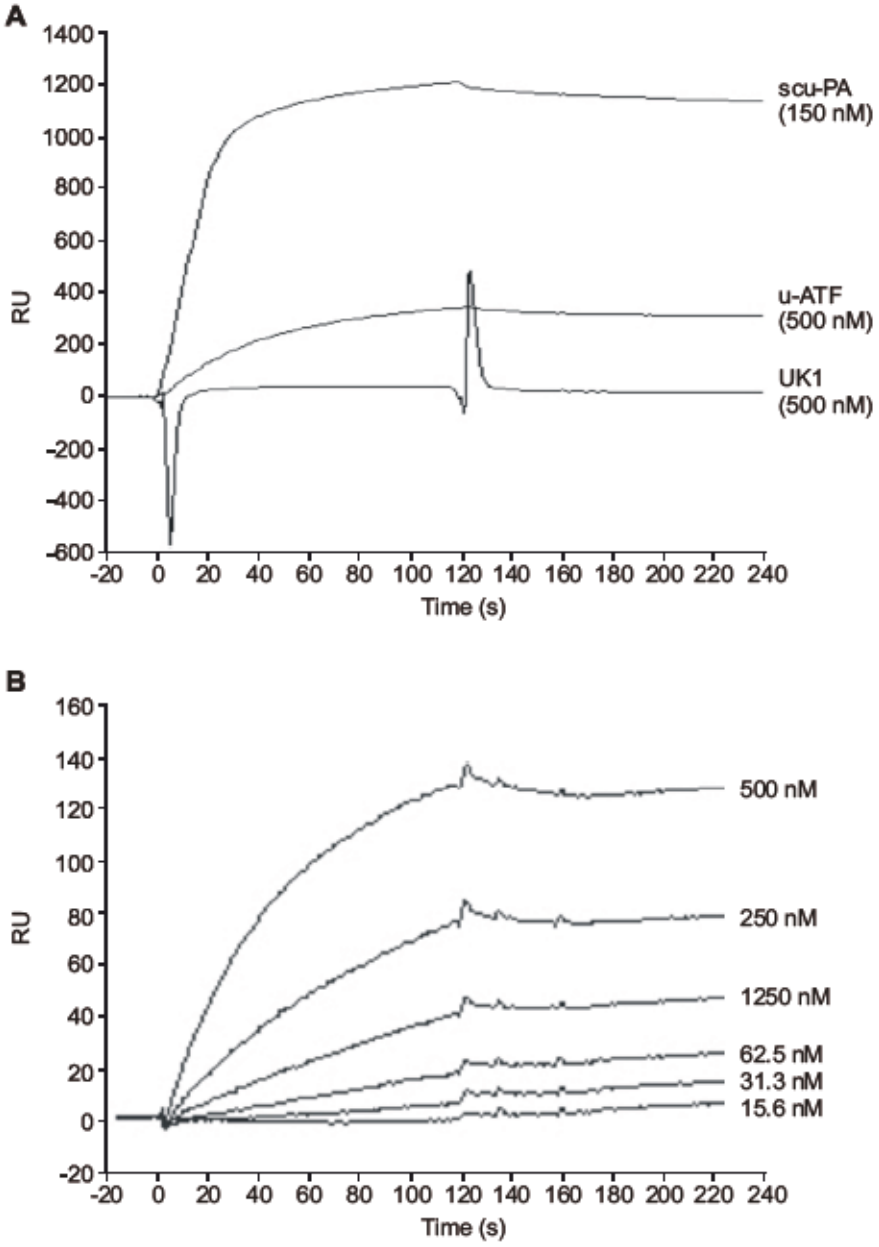

Figure 2. Real-time interaction analysis of binding to immobilized recombinant uPA receptor. Ligand binding curves obtained by automated measurements of surface plasmon resonance (BIACORE technology). A carboxymethyl-type sensor chip was coupled covalently with uPAR as described under "Materials and Methods". Immobilization of uPAR was performed in flow cell 2 while flow cell 1 was immobilized with bovine serum albumin. Samples of scu-PA (A), u-ATF (A, B), and UK-1 (A) were subjected to real-time interaction analysis with the coupled sensor chips, using a Biacore 2000 instrument. Different level of immobilization was applied; (A): for general analysis (R_{max} = 5,100); (B): for kinetics (R_{max} = 780). The samples were diluted in running buffer for serial dilution. Ligand binding was measured at 20°C with passage of the samples through both flow cells. The BIAevaluation software was used for the subtraction of bulk effects (flowcell 1) and a blind curve obtained with buffer alone to compensate for drift.

Inhibition of VEGF-induced endothelial cell migration by UK1 and u-ATF

The migration response of the endothelial cells is more sensitive to VEGF than bFGF (Yoshida *et al.*, 1996), VEGF-induced migration was employed to compare their activitities between u-ATF and UK1. VEGF (2 ng/ml) was used in the low chamber to stimulate the migration of HUVE cell subcultured to passage P3-P4. As shown in Figure 4, purified UK1 and u-ATF also displayed dose-dependent inhibition in a concentration range of 0.01 μg/ml to 10 μg/ml, with IC_{50} of ~1 nM, and 600 nM, respectively. UK1 was about 600 fold more potent than u-ATF. scu-PA itself did not inhibit or potentiate the VEGF-induced endothelial cell migration over a concentration range of 0.01 μg/ml to 10 μg/ml.

Anti-angiogenic activity of UK1 and u-ATF in the chick embryo

To compare the ability of UK1 and u-ATF to inhibit *in vivo* angiogenesis, we used the chick chorioallantoic membrane assay. Purified UK1, and u-ATF

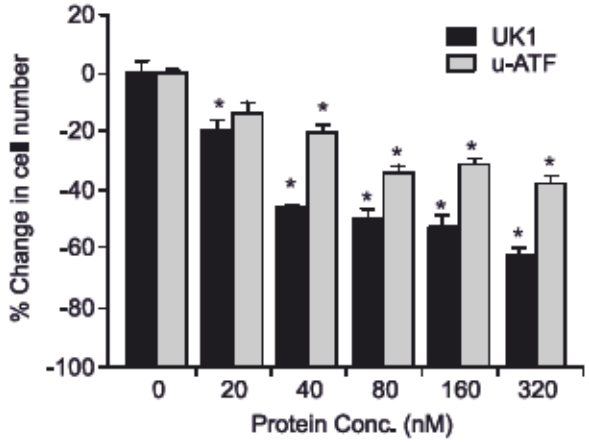

Figure 3. Inhibition of endothleial cell proliferation by UK1 and u-ATF. Anti-endothelial cell proliferative activities were assayed on BCE cell in the presence of 1 ng/ml bFGF for 72 h. Values represent the mean of three determinations (S.E.) as percentages of inhibition. *, $P < 0.01$.

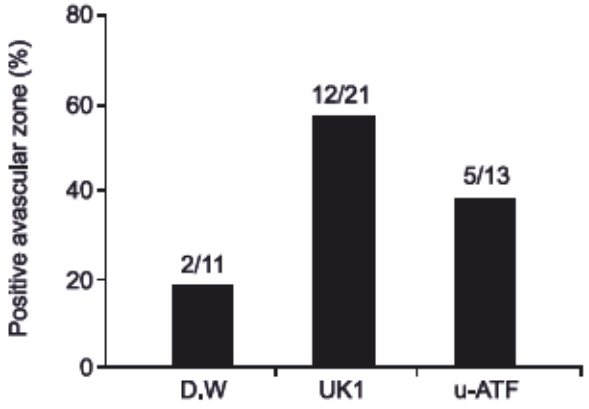

Figure 5. Comparison of *in vivo* anti-angiogenic activities of u-ATF and UK1 in the chick CAM. Discs containing 20 μg of each protein were applied to the CAMs of 4.5-day-old chick embryos. After 48 h, the avascular zone (≥ 5 mm in diameter) of each CAM was examined. Inhibition (%) of treated samples is presented as the number of CAMs with avascular zones over the total number of CAMs (indicated above each column).

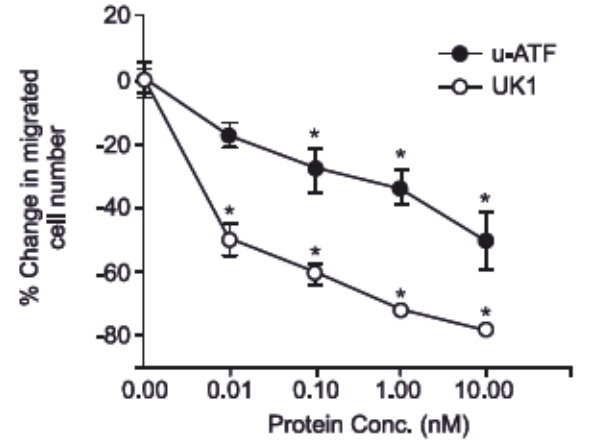

Figure 4. Inhibition of VEGF-induced endothelial cell migration. Anti-migratory activities of UK1 and u-ATF were examined at several doses in a Boyden chamber-based assay. HUVE cells were pre-incubated with test sample at each concentration for 30 min and VEGF (2 ng/ml) was used as a chemoattractant to elicit the migration of cells. Values, given as percentage of inhibition, represent the mean of three determinations (S.E.). *, $P < 0.05$.

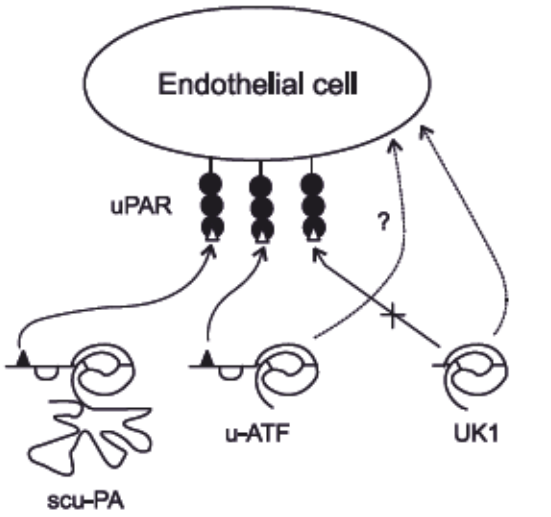

Figure 6. The proposed mechanisms of differential anti-angiogenic acitivity of u-ATF and UK1. The closed triangle represents growth factor-like domain, which interacts with u-PA receptor. u-ATF interacts mainly with u-PAR to block uPA binding, although its kringle domain might interact with endothelial cells via a different mechanism to elicit anti-angiogenic function originated from its kringle structure. UK1 has no binding site for uPAR. The most anti-angiogenic function of UK1 seems to come from different interactions with endothelial cells, rather than uPA/uPAR interaction.

was found to inhibit *in vivo* angiogenesis at the different levels (Figure 5). At doses of 20 μg/embryo, UK1 showed the potent inhibitory activity (57% inhibition), whereas u-ATF showed the marginal inhibitory activity by showing 38% inhibition. Furthermore, the inhibition zone induced by high dose of u-ATF was not so distinct as that of UK1 and its dose-dependent inhibition could not be detected in this assay method. No toxicity was observed in embryos tested.

Discussion

Blockade of cell surface localization of uPA was reported to halt both angiogenesis and tumor growth/or metastasis (Min *et al.*, 1996; Evans *et al.*, 1997; Ignar *et al.*, 1998). Suppression of tumor growth and dissemination by adenovirus-mediated delivery of an uPA/uPAR antagonist, the amino-terminal fragment was

reported to most likely result mainly from an inhibition of angiogenesis (Li *et al.*, 1998; Li *et al.*, 1999; Le Gat *et al.*, 2003). However, the molecular basis of tumor growth and angiogenesis inhibition by uPA antagonists remains to be clarified. uPA has one kringle domain, which shares some homology with those of angiostatin kringles but distinct for being incapable of binding lysine residue. Recent our study demonstrated the kringle domain of uPA, UK1 is an anti-angiogenic molecule (Kim *et al.*, 2003b). In the present study, we examined anti-angiogenic acitivites of UK1 and u-ATF in the identical assay systems. Importantly, our data demonstrated the kringle domain alone is sufficient for potent anti-angiogenic activity *in vitro* and *in vivo*. However, it could not be excluded that ATF can function more effectively in tumor models, considering that ATF domain has a potential cleavage site, Lys^{46}- Ser^{47} for protease to produce UK1 (Bachman, 1994) and that uPA/uPAR interaction might play more important roles in tumor angiogenesis and metastasis.

Additional 45 amino acid-growth factor-like domain in u-ATF seems to divert anti-angiogenic mechanism of kringle domain. Based on the significantly different levels in inhibition of endothelial cell acitivity, u-ATF and UK1 may undergo different mechanisms for eliciting their antiangiogenic activities. In case of angiostatin, which has been studied for 10 years, its cellular and molecular action mechanism is still unclear. Our group reported that UK1 internalize specifically in endothelial cells and it also elicited a transient increase Ca^{2+} flux of more than 2-fold (Kim *et al.*, 2003b). In inhibition of endothelial cell proliferation and migration, UK1 did not show any selectivity on angiogenic factors. The action mechanism of UK1 remains to be elucidated more precisely at the molecular level. In contrast to UK1, u-ATF binds to uPAR with high affinity, but is not able to elicit potent inhibitory activity on endothelial cell proliferation and migration. Since uPAR receptor exists in tumor cells besides endothelial cells, u-ATF seems to act in a non-selective manner, unlike UK1.

One possible explanation for the action mechanism of the differential anti-endothelial cell activities of UK1 and u-ATF can be suggested as shown in Figure 5. The kringle domain alone acts on certain endothelial cell receptor(s) to elicit its potent anti-angiogenic activity, which might be via a mechanism similar to that for angiostatin kringles. By contrast, ATF domain mainly acts as a uPAR antagonist by binding to uPAR, although a minor portion of ATF may bind to the receptor for the kringle domain to elicit endothelial antiproliferative activity.

uPA plays important roles not only in angiogenesis, but also in tumor metastasis. A recombinant adenovirus encoding the non-catalytic ATF of mouse uPA prevents lung carcinoma metastasis (Li *et al.*, 1998) and protects mice in a liver metastasis model of human colon carcinoma (Li *et al.*, 1999). In regard to metastasis, u-ATF can be more effective than UK1. However, a kringle-based inhibitor, angiostatin also suppresses tumor growth and metastasis (O'Reilly *et al.*, 1994). Thus, it will be interesting to examine the efficacy of UK1 and u-ATF molecules in *in vivo* tumor model, in regard to angiogenesis, tumor growth, and metastasis.

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