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Integrin αvß3 is not significantly implicated in the anti-migratory effect of anti-angiogenic urokinase kringle domain

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Abstract. The recombinant kringle domain (UK1) of urokinase-type plasminogen activator (uPA) has been shown to possess anti-angiogenic activity *in vitro* and *in vivo*. It has also been found to inhibit *in vivo* malignant glioma growth. In contrast, direct interaction of the kringle domain of uPA and integrin αvß3 has been reported to be involved in plasminogen and leukocyte activation by uPA. Since integrin αvß3 is involved in tumor angiogenesis, we investigated whether integrin αvß3 is involved in the inhibitory function of UK1 in angiogenesis, by examining its anti-migratory activity. In a modified Boyden chamber assay, the *Pichia*-expressed UK1 dose-dependently inhibited the VEGF-induced migration of human umbilical vein endothelial cells (HUVECs). However, in the absence of growth factor stimulation, soluble UK1 alone did not induce or inhibit HUVEC migration. In cell adhesion, immobilized UK1 promoted HUVEC adhesion and spreading which were compared to BSA. Pretreatment of the anti-αvß3 integrin antibody, significantly inhibited HUVEC binding to immobilized UK1, whereas neither anti-α2ß1 nor anti-α5ß1 integrin antibody had any effect, although pretreatment of the soluble UK1 showed no marked alteration of the binding level of anti-αvß3 antibody to HUVECs in FACS analysis. In a modified Boyden chamber assay, the function blocking antibodies against integrins αvß3, α2ß1 and α5ß1 did not completely prevent the inhibitory effect of UK1 in HUVEC migration. These results suggest that UK1 interacts with integrin αvß3, but its anti-migratory activity on endothelial cells is not significantly mediated by integrin αvß3.

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Introduction

Angiogenesis is a multi-step 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 (1-3). Accordingly, inhibition of angiogenesis has been proposed as a promising strategy for treatment of these diseases (1). Various angiogenesis inhibitors have been reported, such as angiostatin (4), endostatin (5), 16 kDa prolactin (6), kringle of prothrombin 2 (7), kringle 5 of plasminogen (8), kringles 1-2 of the tissue-type plasminogen activator (9), kringle 1 of the urokinase-type plasminogen activator (uPA) (10), kringle 1 and NK4 derived from hepatocyte growth factor (11,12), rhLK68 derived from apolipoprotein (a) (13), tumstatin (14) and endorepellin (15). Several angiogenesis inhibitors have a unique kringle architecture, and kringle domains are composed of ~80 amino acids connected by conserved triple disulfide bonds. The kringle domains derived from various proteins have been reported to elicit anti-angiogenic activity which is different from the parent molecules, but its exact function is unclear (16).

uPA is a serine protease involved in fibrinolysis, and it is implicated in various cellular processes such as inflammation, metastasis and angiogenesis (17-21). uPA induces cell adhesion and chemotactic movement, and its signaling requires its binding to the uPA receptor (uPAR/CD87). In addition, the binding of a uPA-uPAR complex to integrin α5ß1 via uPAR and signal transduction through α5ß1 are required for uPA-induced cell adhesion and migration (22). uPA consists of the three domains of growth factor-like (1-43), kringle (47-135), and carboxyl-terminal protease (136-411). The growth factor domain mediates interaction with the uPA receptor.

In our previous study, we showed that the recombinant kringle domain of uPA (UK1) inhibits the proliferation and migration of endothelial cells (10,23), although UK1 has a low amino acid sequence homology with angiostatin. Recently, we found that UK1 inhibits *in vivo* malignant growth by the suppression of angiogenesis (24). In order to initiate the

study of the UK1 mechanism, we performed experiments and observed that UK1 internalized into the nucleus and induced the transient increase of the Ca^{2+} flux, although possibly by not mediating through interaction with uPAR (10,25). However, its action mechanism is unclear.

In contrast, direct interaction of the kringle domain of urokinase-type plasminogen activator and integrin αvß3 has been reported to enhance plasminogen activation by uPA (26). The kringle domain has been shown to potentiate LPS-mediated neutrophil activation through interaction with the αvß3 integrin (27). Since one of the molecular targets of anti-angiogenic angiostatin, with the first four kringles of plasminogen, has been reported as integrin αvß3 (28), we were very interested in whether integrin αvß3 is an important molecular target of UK1, albeit of low sequence identity with angiostain. In this study, we tested this idea employing an endothelial migration assay, one of the critical steps of angiogenesis.

Materials and methods

Materials. Mouse monoclonal blocking antibodies against integrin αvß3 (LM609), α2ß1 (BHA2.1), or α5ß1 (JBS5) were purchased from Chemicon Inc. (Temecula, CA); anti-mouse-IgG from Zymed Laboratories (San Francisco, CA); VEGF from R&D Systems Inc. (Minneapolis, MN); EGM-2 MV BulletKit medium from Cambrex (Walkersville, MD); fibronectin (FN) and bovine serum albumin (BSA) from Sigma (St. Louis, MO); actin cytoskeleton and focal adhesion staining kit and FITC-conjugated goat anti-mouse antibodies from Chemicon Inc.

Preparation of recombinant UK1. Recombinant UK1 was expressed in *Pichia pastoris*, and purified by S-Sepharose and Q-Sepharose column chromatography as previously described (23). The bacteria endotoxin level of the protein solution was determined to be <0.001 EU/mg using the limulus amebocyte lysate (LAL) assay kit from Cambrex (Walkersville). Purified protein was extensively dialyzed against PBS.

Cell culture. The human umbilical vein endothelial cells (HUVECs) were isolated from human cords as previously described (29). The cells were maintained in M199 (JBI, Daegu, Korea) supplemented with 20% FBS, 30 μg/ml endothelial cell growth supplements (Sigma), 90 μg/ml heparin and 1% antibiotics, at 37˚C in a humidified atmosphere of 5% CO_2. HUVECs used for the experiments were passage 2-8.

Modified Boyden chamber assay. Chemotaxis assay for endothelial cells was carried out by using a 48-well chemotaxis chamber (Neuro Probe, Inc., Cabin John, MD). Micropore polycarbonate membranes (8 μm) were coated with 0.1% gelatin overnight at 4˚C. Detached HUVECs were treated with UK1 or other inhibitors in EBM-2 medium for 30 min, and placed into the upper chamber ($2x10^4$ cells). The lower chamber was added with the buffer containing 0.1% BSA, 90 μg/ml heparin and 2 ng/ml VEGF in EBM-2. The assembled chamber was incubated for 5 h at 37˚C with 5% CO_2. Non-migrated cells on the upper side of the membrane were removed by wiping with a paper towel. The migrated cells were fixed, and stained with Diff-Quik solution (Sysmex Corp., Kobe, Japan). The stained cells were photographed and counted.

Cell adhesion assay. Plates were coated with BSA, FN or UK1 overnight at 4˚C and the remaining non-specific binding sites were blocked by incubating with 1% heat-inactivated BSA (70˚C for 1 h) for 30 min at RT. Serum-starved HUVECs were collected by trypsinization and this reaction was stopped by serum-free EBM-2 media containing a trypsin inhibitor (Sigma) and 2% BSA. The detached cells were washed with serum-free EBM-2 and incubated in the presence or absence of UK1, or integrin antibody for 30 min. The cells ($3x10^4$) were plated on the protein-coated 96-well plates and incubated at 37˚C for 90 min. After non-bound cells were removed by washing with PBS, the remaining bound cells were fixed with 4% paraformaldehyde, and stained with crystal violet. The stained dye was dissolved in 10% acetic acid and quantified by measuring absorbance at 560 nm.

Immunofluorescent analysis of actin stress fibers and focal adhesion. Glass cover slips were coated with BSA, FN or UK1 overnight at 4˚C. Non-specific adhesion sites were blocked by incubating with 1% heat-inactivated BSA. Serum-starved HUVECs collected by trypsinization were plated on the coated plates for 90 min. The cells were fixed, permeabilized and immunostained with anti-vinculin antibody and FITC-conjugated secondary antibody, followed by staining with TRITC-conjugated phalloidin and DAPI. The coverslips were washed and mounted on slide glasses using mounting solution. The fluorescent images were obtained using fluorescence microscopy (Olympus AX70, Tokyo).

Fluorescence activated cell sorting (FACS) analysis. Serum-starved HUVECs were incubated with UK1 (1 μM) in EBM-2 for 30 min at 37˚C, and treated further with anti-α2ß1, -αvß3 or -α5ß1 antibody (10 μg/ml) for 30 min at 4˚C, followed by incubation with the secondary antibody conjugated with Cy3 (Chemicon Inc.). The cells were washed with FACS buffer (2% FBS and 1% sodium azide in PBS), fixed with 1% paraformaldehyde and analyzed on a flow cytometer (FACSCalibur; BD Biosciences). Normal mouse IgG was used as a negative control antibody.

Results

Pichia-derived soluble UK1 inhibits VEGF-induced endothelial cell migration. In a previous study, we showed that *Pichia*-derived UK1 inhibits growth factor-stimulated endothelial cell proliferation as effectively as *E. coli*-derived UK1 (23). Since we have not tested for its anti-migratory activity, we evaluated the inhibitory effect of *Pichia*-derived UK1 in a modified Boyden chamber assay. As shown in Fig. 1A, *Pichia*-derived UK1 potently inhibited VEGF-induced migration in a dose-dependent manner. However, in the absence of growth factor stimulation, soluble UK1 did not significantly induce or inhibit endothelial cell migration on the gelatin matrix (Fig. 1B).

Immobilized UK1 promotes cell adhesion and spreading. Integrin-mediated cell adhesion is a required step for cell

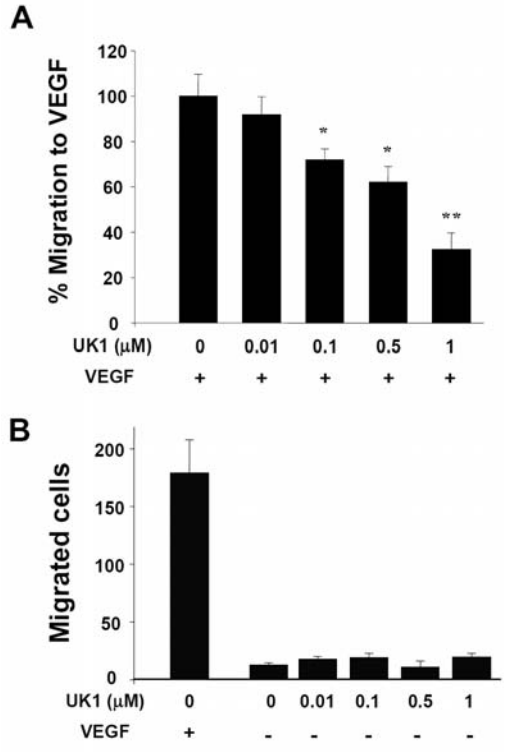

Figure 1. Inhibitory effect of UK1 on VEGF-induced migration. However, no effect on the basal movement in the absence of the growth factor occurred. Chemotaxis assay for endothelial cells was carried out by using a 48-well chemotaxis chamber. HUVECs were pretreated with soluble UK1 at the indicated concentration for 30 min at 37˚C. The cells were allowed to migrate for 5 h in the presence or absence of 2 ng/ml VEGF. Cell migration is presented as a relative percentage compared with the VEGF-induced control group (A). Each value represents the mean ± SE. *p<0.05 and **p<0.01, compared with the VEGF-induced control group.

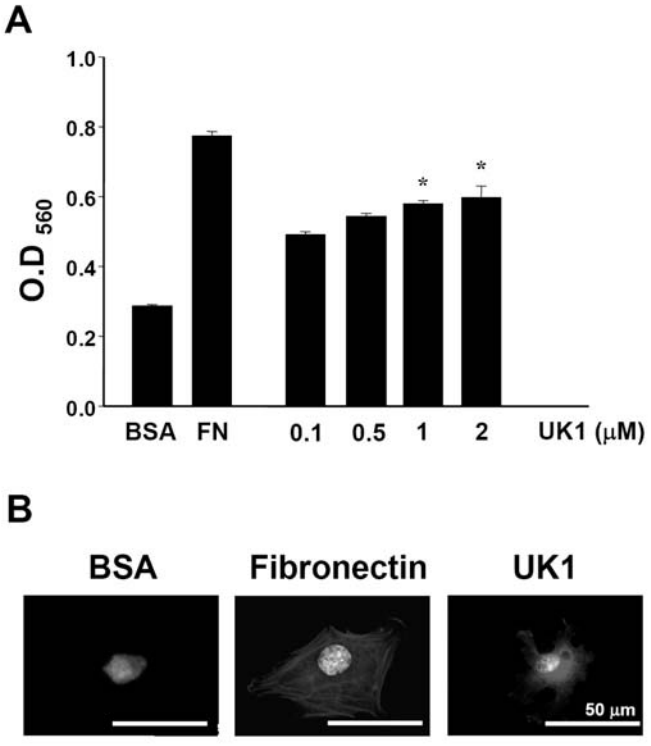

Figure 2. Immobilized UK1 promotes cell adhesion and spreading. (A) HUVECs were seeded on 96-well plates coated with BSA (1%), fibronectin (10 μg/ml), or the indicated concentration of UK1, and incubated for 90 min. After removing non-bound cells by washing with PBS, the attached cells were stained with crystal violet, and the stained dye was dissolved, followed by an absorbance measurement at 560 nm (mean ± SE). *p<0.05, compared with the UK1 (0.1 μM)-treated group. (B) HUVECs were attached to the plates coated with BSA (1%), fibronectin (100 μg/ml), or UK1 (100 μg/ml) for 90 min. Actin stress fibers of the attached cells were visualized by staining with TRITC-conjugated phalloidin. The representative cell image is shown. Red fluorescence is shown as a white color. Magnification, x400.

migration. In addition, the kringle domain of uPA has been shown to directly interact with integrin αvß3. HUVECs express integrin subunits ß1, α2, α5 and αv (30). Thus, we examined whether UK1 binds to HUVECs and whether its binding involves integrins including αvß3. We initially tested, in cell adhesion assay, whether HUVECs interact with UK1 or not. In this assay, we found that adhesion of HUVECs to UK1-coated dishes was increasingly dose-dependent (Fig. 2A). When the actin filaments of attached cells were visualized by fluorescence, the cells attached to immobilized UK1 did not form apparent actin stress fibers compared to the cells attached to fibronectin (Fig. 2B). Accordingly, focal adhesion formation visualized by anti-vinculin antibody staining was manifested in the cells attached to fibronectin, but was rarely observed in the cells attached to UK1 (data not shown). However, UK1-attached cells were more disseminated than those attached to BSA, which maintained their small round shape. Therefore, we concluded that immobilized UK1 promotes endothelial cell adhesion and spreading.

Binding of HUVECs to immobilized UK1 is inhibited by anti-αvβ3 integrin antibody. Since HUVECs adhere and disseminate to immobilized UK1, we examined whether integrin blocking antibodies affect the attachment of HUVECs to immobilized UK1. The integrins α1ß1, α2ß1, α5ß1 and αvß3 are up-regulated during tumor angiogenesis (31) and integrin α1 is rarely expressed in HUVECs (30). Thus, we examined the effects of anti-αvß3, -α2ß1 and -α5ß1 antibodies on HUVEC binding to immobilized UK1. As shown in Fig. 3A, anti-αvß3 integrin antibody treatment significantly inhibited the HUVEC binding to immobilized UK1, whereas the anti-α5ß1 and -α2ß1 antibodies did not show any inhibition in cell adhesion. This result corresponds with the previous data showing that recombinant soluble αvß3 binds to the immobilized uPA kringle (26). When the cells were pretreated with soluble UK1 prior to cell adhesion, the cellular attachment on immobilized UK1 was decreased significantly, suggesting that HUVEC adhesion on immobilized UK1 is not from non-specific binding.

We examined whether soluble UK1 was able to bind to integrins αvß3, α2ß1 or α5ß1. In this experiment, we compared the binding level of each monoclonal integrin antibody between untreated or UK1-pretreated cells (37˚C, 30 min) by FACS analysis, to assess the level of integrins bound to UK1, which would be accessible to the anti-integrin antibodies (Fig. 3B). However, the pretreatment of UK1 did not show

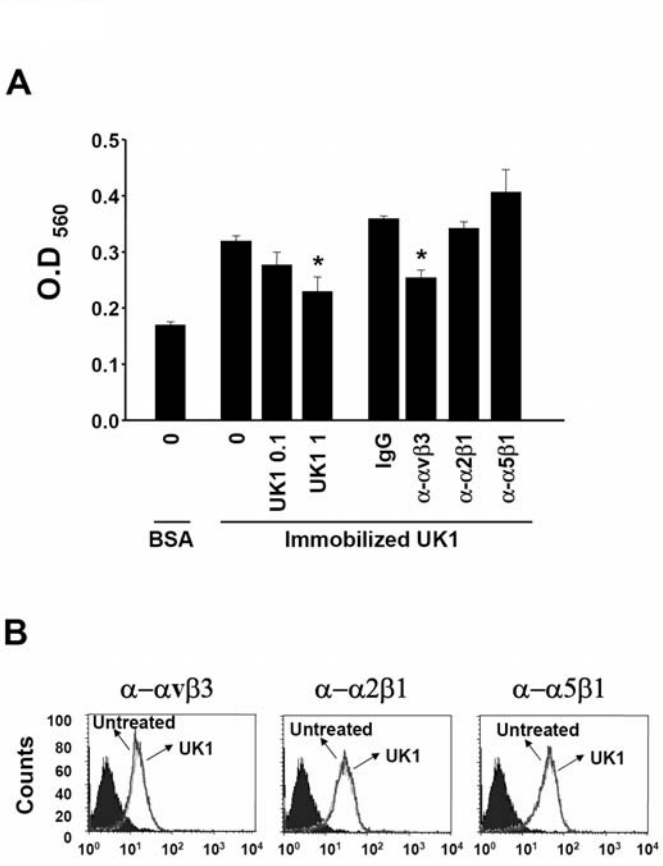

Figure 3. Adhesion of HUVECs to immobilized UK1 is blocked by anti-αvß3 integrin antibody. (A) HUVECs were incubated in the presence or absence of UK1, or the integrin antibody (10 μg/ml for each integrin antibody) for 30 min. The cells ($3x10^4$) were plated on immobilized UK1 (2 μM) and incubated for 90 min at 37˚C. The attached cells were stained with crystal violet, and the stained dye was dissolved, followed by an absorbance measurement at 560 nm (mean ± SE). *p<0.05, compared with the relevant control group. (B) HUVECs were incubated with UK1 (1 μM) for 30 min at 37˚C, and treated further with anti-αvß3, -α2ß1 or -α5ß1 antibody (10 μg/ml) for 30 min at 4˚C, followed by incubation with the secondary antibody conjugated with Cy3. The cells were washed, fixed, and analyzed by flow cytometry. Mouse IgG was used as a negative control. The dark area indicates mouse IgG control, the gray line UK1-untreated cells and the black line UK1-treated cells.

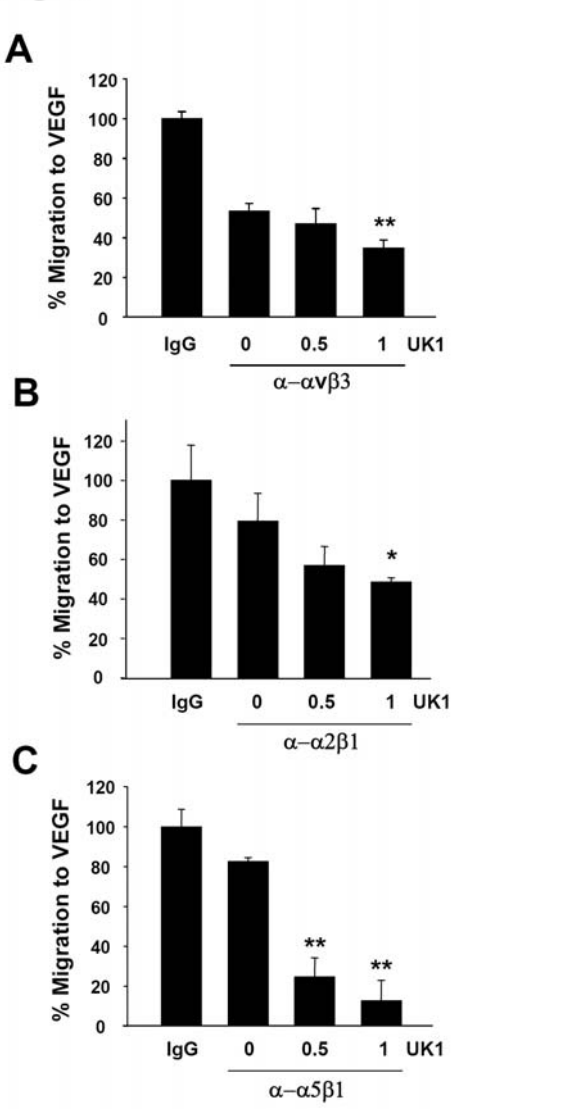

Figure 4. Anti-migratory effect of UK1 is not prevented by integrin αvß3. HUVECs were pretreated with either anti-αvß3 (A), anti-α2ß1 (B) or anti-α5ß1 (C) integrin antibody for 30 min at 37˚C and then treated with UK1 for another 30 min. The cells were allowed to migrate in the presence of VEGF (2 ng/ml) for 5 h. Cell migration is presented as a relative percentage compared with the relevant control group. *p<0.05 and **p<0.01, compared with the relevant VEGF-induced, IgG-treated control group.

any significant change in the binding level of the tested integrin antibodies. One possible explanation for the lack of change in the binding of anti-αvß3 integrin antibody on UK1 treatment is that the binding site of the small molecule UK1 is different from the binding site of anti-αvß3 integrin antibody or that the binding of the small molecule UK1 does not hinder or alter the binding site of anti-αvß3 integrin antibody. Taken together, we concluded that integrin αvß3 may be involved in UK1 binding to HUVECs.

Anti-αvß3 integrin antibody does not completely prevent the anti-migratory effect of UK1. Since the anti-αvß3 integrin antibody blocks HUVEC binding to immobilized UK1, we examined the effects of anti-αvß3 integrin antibody on the anti-migratory effect of UK1, together with the anti-α2ß1 and -α5ß1 integrin antibodies. When the cells were pretreated with anti-integrin antibody at each optimal concentration prior to UK1 treatment, notably, the tested integrin antibodies including anti-αvß3 did not completely prevent the inhibitory effect of UK1 on VEGF-induced endothelial cell migration, despite showing different levels of prevention (Fig. 4). Therefore, we concluded that integrin αvß3 may not be significantly involved in the anti-migratory effect of UK1.

Discussion

Recombinant UK1 inhibits angiogenesis *in vivo*, and *in vitro* (10,23). We have shown that UK1 inhibits malignant glioma growth by suppression of angiogenesis (24). In that study, we showed that UK1 inhibits endothelial cell proliferation and tube formation, but not the proliferation of glioma cells. Various kringle domains with a wide range of sequence identities between the kringles (32.5-83.3% to plasminogen

SPANDIDOS PUBLICATIONS) have been reported to inhibit angiogenesis and growth, although they exhibit different levels of anti-angiogenic activities. However, their action mechanisms remain unclear.

In this study, we found that UK1 inhibited VEGF-induced endothelial cell migration and that soluble UK1 alone did not induce or inhibit endothelial cell migration on the gelatin matrix. As reported by other researchers, we showed that UK1 binding to HUVECs involves integrin αvß3, but not integrins α2ß1 and α5ß1. However, we found that the anti-αvß3, anti-α2ß1 and anti-α5ß1 integrin antibodies did not completely prevent the anti-migratory effect of UK1. We concluded that integrin αvß3 may not play a major role in the inhibition of endothelial cell migration by UK1, suggesting that other mechanisms or other potential molecular targets may be involved in the anti-angiogenic activity of UK1.

In a previous study, we compared the inhibitory effects between the amino-terminal fragment of uPA (u-ATF) and UK1 (25). The u-ATF domain has one additional functional epitope, a growth factor-like domain, which interacts with the uPA receptor. Notably, u-ATF exhibited far less sensitivity than did UK1 in VEGF-induced migration. From that study, we assumed that UK1 and u-ATF have different molecular targets, and anti-angiogenic activity of UK1 is not related to the uPA receptor. We also observed that the internalization of UK1 followed by the translocation from cytosol to the nucleus within 30 min occurs in endothelial, but not in non-endothelial cells (10). The present study provided some evidence that integrin αvß3 may not be the major receptor of UK1. Thus, how UK1 internalizes in cells remains unclear.

Apart from the anti-angiogenic activity, other researchers reported on the role of the kringle domain of uPA. They presented the positive role of the kringle domain in uPA molecule by showing that direct interaction of the kringle domain of uPA and integrin αvß3 induces signal transduction and enhances plasminogen activation by uPA (26). The kringle domain of uPA has been reported to potentiate LPS-mediated neutrophil activation through interaction with integrin αvß3 (27). Integrin αMß2 also directly binds to the kringle domain of uPA in neutrophil (32). Since endothelial cells do not express αMß2, we focused on integrin αvß3 in order to investigate a potential molecular target of UK1. Unlike the immobilized kringle domain of uPA that induced migration of CHO cells in an αvß3-dependent manner (26), the soluble kringle domain appeared bound to integrin αvß3, but did not induce endothelial cell migration. The kringle domain within uPA may play an important role in uPA activation. However, the soluble kringle domain alone behaves differently by showing anti-angiogenic activity, unlike uPA. Accordingly, integrin αvß3 may not be dominantly involved in the anti-angiogenic activity of UK1.

A potential target of the anti-angiogenic action of angiostatin has been proposed as integrin αvß3 (28). The uPA kringle domain binding to integrin αvß3 can be antagonized by angiostatin (26). Our study showed that anti-αvß3 integrin antibody inhibited HUVEC binding to immobilized UK1. Therefore, UK1 may act as an integrin αvß3 antagonist as does angiostatin, and may partially play a role in the anti-angiogensis of UK1. However, the anti-αvß3 integrin antibody did not completely prevent the anti-migratory effect of UK1. This result suggests that other plausible major molecular targets of UK1 exist. For a complete explanation of the mechanism of action of UK1 and elucidation of an important functional epitope, which provides the basis for the development of new anti-angiogenic and anti-tumoric agents, further studies are necessary.

Acknowledgements

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