# Antimigratory effect of TK1-2 is mediated in part by interfering with integrin $\alpha_2\beta_1$

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## Abstract

**The recombinant two kringle domain of human tissue-type plasminogen activator (TK1-2) has been shown to inhibit endothelial cell proliferation, angiogenesis, and tumor cell growth despite of sharing a low amino acid sequence homology with angiostatin. Here, we explored a possible inhibitory mechanism of action of TK1-2 by focusing on antimigratory effect. TK1-2 effectively inhibited endothelial cell migration induced by basic fibroblast growth factor or vascular endothelial growth factor in a dose-dependent manner and tube formation on Matrigel. It blocked basic fibroblast growth factor–induced or vascular endothelial growth factor–induced phosphorylation of extracellular signal-regulated kinase 1/2 and formation of actin stress fibers and focal adhesions. Interestingly, TK1-2 alone induced the weak phosphorylation of focal adhesion kinase, whereas it inhibited focal adhesion kinase phosphorylation induced by growth factors. When immobilized, TK1-2 promoted adhesion and spreading of endothelial cells compared with bovine serum albumin. However, treatment with anti-$\alpha_2\beta_1$ blocking antibody markedly diminished endothelial cell adhesion to immobilized TK1-2 compared with anti-$\alpha_v\beta_3$ or anti-$\alpha_5\beta_1$ antibody. Pretreatment of soluble TK1-2 also altered the binding level of anti-$\alpha_2\beta_1$ antibody to endothelial cells in fluorescence-activated cell sorting analysis. Indeed, a blocking antibody against integrin $\alpha_2\beta_1$ or knocking down of integrin $\alpha_2$ expression prevented the inhibitory effect of TK1-2 in cell migration. Therefore, these results suggest that TK1-2 inhibits endothelial cell migration through inhibition of signaling and cytoskeleton rearrangement in part by interfering with integrin $\alpha_2\beta_1$. [Mol Cancer Ther 2008;7(7):2133–41]**

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## Introduction

Tumor growth and metastasis are critically dependent on angiogenesis and formation of new blood vessels (1). Therefore, inhibition of angiogenesis has been a promising strategy for cancer therapy. A lot of efforts have been driven to discover new angiogenesis inhibitors, and several endogenous protein fragments have been discovered to effectively inhibit angiogenesis differently from parent molecules (2, 3).

Tissue-type plasminogen activator is a serine protease that activates fibrinolysis through the conversion of plasminogen to plasmin (4). It consists of five distinct structural domains that contain a finger domain, an epidermal growth factor–like domain, two kringle domains, and a COOH-terminal proteolytic domain. Interestingly, the recombinant protein comprising only two kringle domain of tissue-type plasminogen activator (TK1-2) has been shown to have antiproliferative effect on endothelial cells in our previous studies (5). It has also been shown to inhibit *in vivo* angiogenesis in chick chorioallantoic membrane and tumor growth in lung or colon cancer xenograft murine models (5–7). Later, a domain deletion mutant of tissue-type plasminogen activator containing kringle 2, a thrombolytic agent named Reteplase, has been found to elicit antiangiogenic activity by other researchers, providing new mechanistic insights into the bleeding complications of this drug (8). They also identified the kringle domain 2 alone as a novel molecule for antiangiogenic therapy. Recently, we also reported that TK1-2 inhibits adhesive differentiation of endothelial progenitor cells and their contribution to tumor vessel formation *in vivo* (9). Therefore, we were interested in investigating the mechanism of action of TK1-2, because the effective inhibitory effects of TK1-2 *in vitro* and *in vivo* were clearly shown.

Generally, the kringle domains are composed of ~80 amino acids connected by conserved triple disulfide bonds (10). They are found in various proteins, such as plasminogen, prothrombin, apolipoprotein(a), hepatocyte growth factor, and urokinase-type plasminogen activator, besides tissue-type plasminogen activator. Although the exact physiologic function of kringle domains has not been elucidated, there have been several reports that the kringle domains derived from various proteins elicit antiangiogenic activity *in vitro* and *in vivo* differently from parent molecules (5, 6, 11–17). With a wide range of sequence identities between the kringle domains (32.5-83.8% to plasminogen kringle 5), these kringle domains display various levels of *in vitro* anti-endothelial cell and antiangiogenic activities. However, their mechanisms of action are unclear (18). For angiostatin consisting of the first one to four kringles of plasminogen, several putative molecular targets including F1-F0 ATP synthase, integrin $\alpha_v\beta_3$, and

angiomotin have been suggested to explain in part its possible mechanism of action (19–21). In the case of plasminogen kringle 5, it has been shown to induce apoptosis of endothelial and tumor cells through surface-expressed glucose-regulated protein 78 (22).

Angiogenesis is a complex multistep process that includes endothelial cell proliferation, migration, and differentiation (23). Integrins are required for cell proliferation, survival, and migration and are important for the growth of new blood vessels because endothelial cells are anchorage dependent. The growth of new blood vessels is a dynamic yet highly regulated process that depends on coordinated signaling by growth factor and integrin receptors (24). Indeed, integrin antagonists that prevent binding of $\alpha_2\beta_1$ (25), $\alpha_v\beta_3$ (26), and $\alpha_5\beta_1$ (27) to extracellular matrix suppress tumor growth via angiogenesis inhibition. The $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins provide critical support for vascular endothelial growth factor (VEGF) signaling and endothelial cell migration (25).

Because TK1-2 effectively inhibited endothelial cell migration, we investigated the mechanism of action of TK1-2 for antiangiogenic activity at cellular and molecular levels by focusing on its antimigratory activity. Our present studies show that integrin $\alpha_2\beta_1$ is a potential target of antiangiogenic TK1-2.

## Materials and Methods

### Materials

Basic fibroblast growth factor (bFGF) and VEGF were purchased from R&D Systems. EGM-2 MV BulletKit medium was from Cambrex. Actin cytoskeleton and focal adhesion staining kit and FITC-conjugated goat anti-mouse antibody were from Chemicon. Anti-phospho-focal adhesion kinase (FAK; pY397) and anti-FAK antibodies were from Upstate Biotechnology. Anti-phospho-extracellular signal-regulated kinase (ERK) 1/2 and anti-ERK1/2 antibodies were from Cell Signaling Technology. Mouse monoclonal blocking antibodies against $\alpha_2\beta_1$ (BHA2.1), $\alpha_5\beta_1$ (JBS5), or $\alpha_v\beta_3$ (LM609) integrin were from Chemicon. Anti-rabbit and anti-mouse horseradish peroxidases were from Santa Cruz Biotechnology. Anti-mouse IgG from Zymed Laboratories.

### Preparation of Recombinant TK1-2 and Plasminogen Kringles 1-3

The recombinant TK1-2 was prepared as described previously (6). The recombinant plasminogen kringles 1-3 (PK1-3) was expressed in *Pichia pastoris* as a nonglycosylated protein as follows. A 762-bp DNA fragment encoding amino acids spanning from $Ser^{355}$ to $Ser^{1116}$ of human plasminogen was amplified by PCR using the PK1-3 DNA fragment (28), *Pfu* DNA polymerase (Stratagene), a forward primer A [5′-CAGTATCGATCTCAGAGTGCAAGACTGGG-3′ (*Cla*I)], and a reverse primer B [5′-CTGATCTAGACTAGGAGTCACAGGACGG-3′ (*Xba*I)]. The PCR product was digested with *Cla*I and *Xba*I, ligated into pPICZα-C linearized with the same enzyme set, and then transformed into *Escherichia coli* TOP10 F. After that, site-directed mutagenesis was done on the PK1-3 DNA to create the $Asn^{976}$ to $Glu^{976}$ mutation. Briefly, the oligonucleotide primers containing the desired mutation were extended during thermal cycling by using Native *Pfu* DNA polymerase (Stratagene). Used primers are forward primer A (5′-GACCCCTCACACACATGAGAGGACACCAGAAAACTTC-3′) and reverse primer B (5′-GAAGTTTTCTGGTGTCCTCTCATGTGTGTGAGGGGTC-3′). The PCR product was treated with *Dpn*I and transformed into *E. coli* strain XL10-Gold (Stratagene). After confirming the expected mutation by DNA sequencing, the plasmid expressing nonglycosylated PK1-3 (pPICZα-NE-PK1-3) was linearized with *Sac*I enzyme (Roche Molecular Biochemicals) and used for homologous recombination into *Pichia* strain X-33 by electroporation. Selection, expression, and purification were done by the similar method as described for TK1-2 previously (6).

### Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from human cords as described previously (29) and cultured in M199 (JBI) supplemented with 20% fetal bovine serum, 30 μg/mL endothelial cell growth supplements (Sigma), 90 μg/mL heparin, and 1% antibiotics at 37°C in a 5% $CO_2$ atmosphere.

### Wound Migration Assay

HUVEC were cultured in 1% gelatin-coated 24-well plates containing EGM-2 medium until confluence and then washed with PBS and starved in serum-free EBM-2 medium for 4 h. The confluent cells were scraped with a pipette tip and washed with PBS to remove cellular debris. The cells were treated with TK1-2 in EBM-2 medium for 30 min and then incubated in the presence of 1% fetal bovine serum and bFGF. Migration of the cells into the wound area was allowed for 8 h at 37°C. The wound area was photographed with an Olympus C-3030 digital camera at 0 and 8 h, and migrated cells were counted. All the experiments were done in triplicate.

### Modified Boyden Chamber Assay

Chemotaxis assay for endothelial cells was done by using a disposable Transwell (Costar) or a 48-well chemotaxis chamber (Neuro Probe). Using the Transwell, the membrane was coated with 0.1% gelatin (Sigma). Serum-starved cells were detached from cultured dishes and washed with serum-free M199. The cells were suspended in $4 \times 10^5$ per mL in M199 medium containing heparin and treated with TK1-2 for 30 min at 37°C. Then, the cells ($4 \times 10^4$) were seeded into the upper chamber, and it was placed into the lower chamber filled with 600 μL migration buffer containing 0.1% bovine serum albumin (BSA), heparin, VEGF, and TK1-2 in M199 medium.

Using the 48-well chemotaxis chambers, 8 μm micropore polycarbonate membranes were coated with gelatin overnight at 4°C. Detached HUVEC were treated with TK1-2 or other inhibitors in EBM-2 medium for 30 min and then seeded into upper chamber ($2 \times 10^4$ cells). The low chamber was added with the buffer containing 0.1% BSA, heparin, and growth factor in EBM-2. After assembling the chambers, the incubation was executed for 5 h at 37°C to

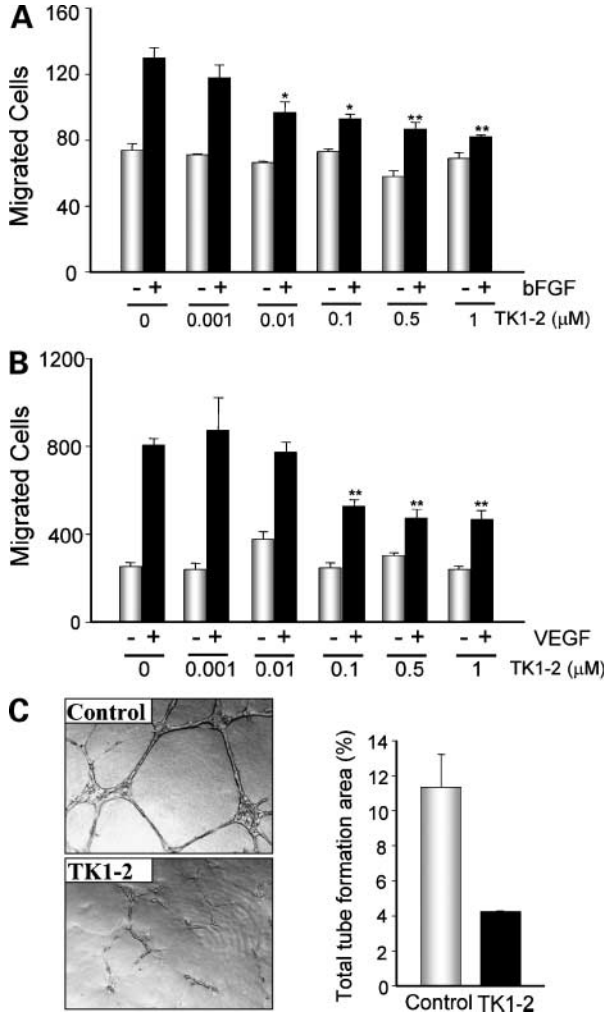


**Figure 1.** Inhibitory effect of TK1-2 on endothelial cell migration and tube formation. **A,** cell motility was assessed in a wound migration assay. HUVEC monolayer was wounded with a pipette tip and then incubated in the medium containing 1% fetal bovine serum with or without 3 ng/mL bFGF for 8 h after pretreatment of TK1-2 for 30 min. **B,** cell motility was assessed by a chemotaxis assay. HUVEC were incubated with TK1-2 for 30 min and placed in the upper chamber. The bottom chamber contained 2 ng/mL VEGF. Then, the cells were allowed to migrate for 5 h. Mean ± SE. **C,** HUVEC were incubated on Matrigel in the absence (control) or presence of TK1-2 for 18 h. Magnification, ×100. The graph presents total tube formation area obtained by using Image J program (http://rsb.info.nih.gov/ij/). *, $P < 0.05$; **, $P < 0.005$, compared with growth factor alone-treated control.

allow the cells to migrate. The migrated cells were fixed and stained with Diff-Quik solution (Sysmex). The stained cells were photographed and counted. All the experiments were done in triplicate.

### Tube Formation Assay

Matrigel basement membrane matrix (400 μL; BD Bioscience) was added to each well of chilled 24-well plates and incubated for 30 min at 37°C. HUVEC ($4 \times 10^4$) were treated with TK1-2 (0.5 μmol/L) for 30 min and then added to the top of solidified Matrigel in the well. During incubation, tube formation was observed, and five representative fields were photographed at 18 h.

### Western Blot Analysis

Serum-starved HUVEC were treated with TK1-2 for 30 min and then incubated with bFGF (3 ng/mL) or VEGF (2 ng/mL) for the indicated time. Then, the cells were washed with ice-cold PBS and lysed. The lysates were centrifuged at 14,000 rpm for 30 min, and the supernatant was separated in SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane, and the membrane was blocked with 5% skim milk. Blots were incubated with specific primary antibodies and incubated with horseradish peroxidase–conjugated secondary antibodies. The immunoreactive bands were visualized using a chemiluminescent substrate (ECL kit; Amersham Pharmacia Biotech). Signals were quantified densitometrically using Multi Gauge V3.0 software version (Fuji Photo Film).

### Immunofluorescence Analysis of Actin Stress Fibers and Focal Adhesions

HUVEC were seeded on gelatin-coated glass coverslips in EGM-2 medium. After 24 h, the cells were starved in serum-free EBM-2 medium for 4 h and treated with TK1-2 for 30 min. Then, the cells were treated with 3 ng/mL bFGF for 30 min. After washing with PBS, the cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100 for 3 min. Nonspecific protein binding sites were blocked with 1% BSA for 30 min, and the cells were incubated with monoclonal antibody against vinculin. After washing, the cells were incubated with FITC-labeled secondary antibody. For staining actin cytoskeleton, the cells were incubated with TRITC-conjugated phalloidin for 3 h at room temperature followed by staining with 4′,6-diamidino-2-phenylindole. The coverslips were then washed and mounted on glass slides using mounting solution. The fluorescent images were obtained using a fluorescence microscopy (Olympus AX70).

### Cell Adhesion Assay

Plates (96 wells) were coated with the indicated concentration of proteins for 16 h at 4°C. Nonspecific adhesion sites were saturated by incubating with the 1% heat-inactivated BSA (70°C for 1 h) at room temperature for 30 min. Serum-starved HUVEC were collected by trypsinization. These reactions were stopped by serum-free EBM-2 medium containing trypsin inhibitor (Sigma) and 2% BSA. The detached cells were washed with serum-free EBM-2 and incubated in the presence or absence of TK1-2 or integrin antibody for 30 min. Then, the cells ($1 \times 10^4$-$3 \times 10^4$) were plated on the coated plates and incubated at 37°C for 90 min. After washing with PBS, remaining attached cells were fixed with 4% paraformaldehyde and stained with crystal violet. The stained dye was dissolved in 10% acetic acid followed by measurement of absorbance at 560 nm.

### Fluorescence-Activated Cell Sorting Analysis

Serum-starved HUVEC collected by trypsinization were incubated with TK1-2 in EBM-2 for 30 min at 37°C and further treated with anti-$\alpha_2\beta_1$, anti-$\alpha_v\beta_3$, or anti-$\alpha_5\beta_1$ antibody (10 μg/mL) for 30 min at 4°C followed by incubation with secondary antibody conjugated with Cy3

(Chemicon). The cells were washed with fluorescence-activated cell sorting buffer (2% fetal bovine serum and 1% sodium azide in PBS), fixed with 1% paraformaldehyde, and then analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Mouse IgG was used as a negative control.

### Small Interfering RNA – Mediated Knockdown of Integrin $\alpha_2\beta_1$ Expression

Validated $\alpha_2$ integrin small interfering RNA (siRNA) and negative control siRNA were purchased from Ambion. HUVEC were plated to a 50% confluence in antibiotic-free EGM-2 medium. siRNA and LipofectAMINE RNAiMAX (Invitrogen) was diluted into Opti-MEM I reduced serum medium (Invitrogen) according to the instruction of the supplier. The diluents were mixed and incubated for 20 min at room temperature for formation of transfection complex. HUVEC were incubated with the transfection complex to a final RNA concentration of 10 nmol/L for 4 h and then replaced by fresh medium. After incubation of 48 h, the cells were subjected to a migration assay, a Western blot analysis, and fluorescence-activated cell sorting analysis.

## Results

### TK1-2 Inhibits Endothelial Cell Migration and Capillary Formation

Because cell migration constitutes an important process in vessel formation, we tested if TK1-2 could inhibit endothelial cell migration induced by angiogenic growth factors, VEGF and bFGF. TK1-2 itself could not affect cell migration in the absence of growth factor. However, under bFGF-stimulated condition, TK1-2 dose-dependently inhibited the migration of HUVEC in a wound migration assay (Fig. 1A). In the experiment of chemotactic movement induced by VEGF, TK1-2 also potently inhibited HUVEC migration in a dose-dependent manner (Fig. 1B). In addition, TK1-2 inhibited VEGF-induced migration of human microvascular dermal endothelial cells (data not shown). When HUVEC were pretreated with Z-VAD-fmk, a pan-caspase inhibitor, before TK1-2 treatment, such pretreatment did not affect the antimigratory effect of TK1-2 (data not shown), and under migration assay conditions, we could not observe cell death due to TK1-2 treatment, suggesting that antimigratory effect of TK1-2 is not due to induction of apoptosis during the experiments. Thus, all the data indicate that TK1-2 effectively inhibits endothelial cell migration. When we examined the effect of TK1-2 on *in vitro* tube formation on Matrigel for 18 h, TK1-2 prevented markedly formation of capillary morphogenesis (Fig. 1C). The expanding and elongation of HUVEC cytosol seemed defective in the presence of TK1-2 during the process of capillary morphogenesis with markedly hindered cellular attachment and migration.

### TK1-2 Perturbs Migration Signaling Pathways: ERK and FAK

As activation of mitogen-activated protein kinase is involved in endothelial cell migration in response to angiogenic growth factors (30), we examined the effect of TK1-2 on ERK1/2 activation induced by growth factor in HUVEC. The pretreatment with TK1-2 before growth factor stimulation reduced phosphorylation of ERK1/2 induced by bFGF or VEGF compared with TK1-2-untreated cells (Fig. 2A and B, *bottom*). This result corresponds with the notion that ERK pathway inhibitors PD98059 and U0126 inhibit the phosphorylation of ERK and the migration of HUVEC (31, 32).

FAK, a 125-kDa protein kinase, has also been known as an important regulator in the changes of actin cytoskeleton that is a prerequisite for cell migration (33). Phosphoryla-

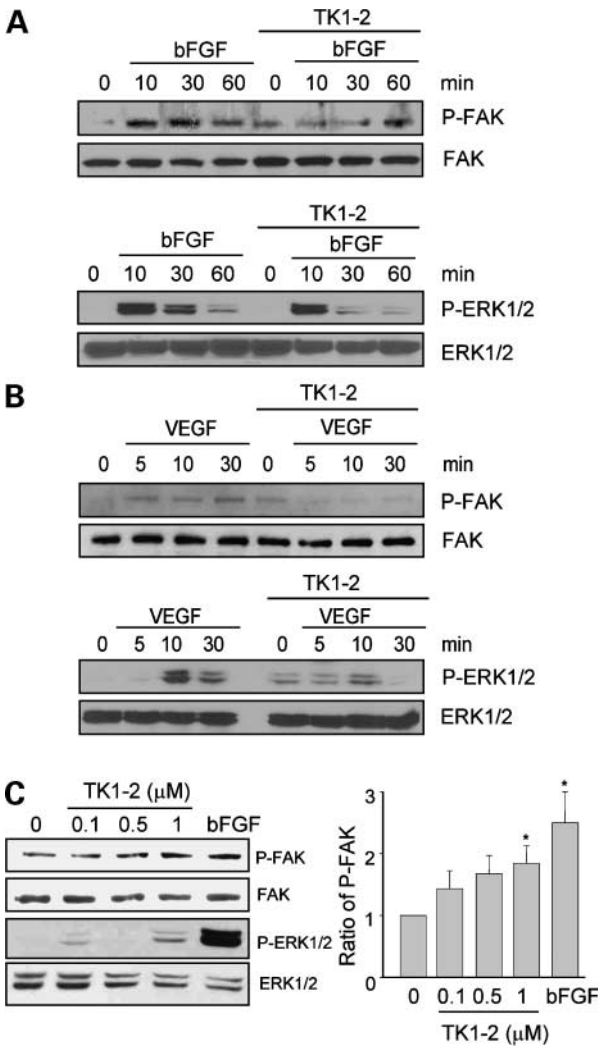


**Figure 2.** Inhibitory effects of TK1-2 on activation of ERK1/2 and FAK. **A** and **B,** HUVEC were treated with TK1-2 (0.5 μmol/L) for 30 min and then stimulated with bFGF (**A**) or VEGF (**B**) for the indicated time. **C,** HUVEC were treated with TK1-2 for 30 min at the various concentrations. After incubation, the cell lysate was immunoblotted to detect phospho-ERK1/2 or phospho-FAK. The blot was stripped and reprobed for total ERK1/2 or FAK detection. The graph presents densitometric analysis of the blot. The density of each protein band was normalized with that of FAK. Mean ± SE of three independent Western blot experiments. *, $P < 0.05$, compared with control.

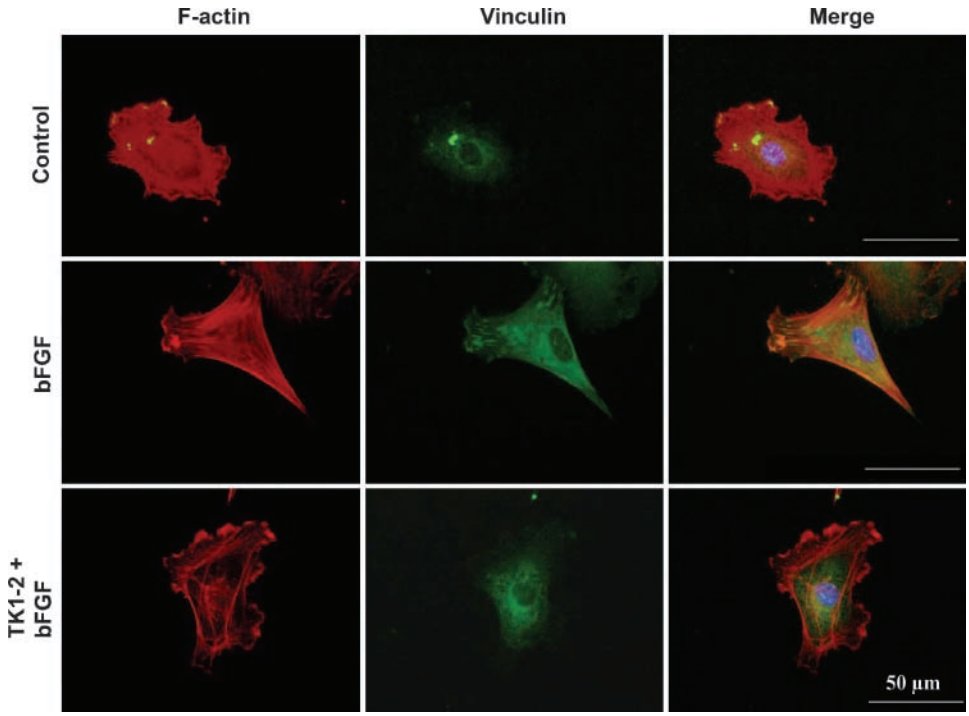


**Figure 3.** Inhibition of formation of actin stress fibers and focal adhesions by TK1-2. HUVEC were pretreated with TK1-2 (0.5 μmol/L) for 30 min and incubated in the presence of bFGF for 30 min. Then, the cells were immunostained with anti-vinculin antibody and stained with TRITC-conjugated phalloidin and 4′,6-diamidino-2-phenylindole. Magnification, ×400.

tion of FAK at Y397 site is required for FAK function in promoting both growth factor–mediated and integrin-mediated cell motility (34). Thus, we examined the effect of TK1-2 on phosphorylation of FAK along with ERK1/2. TK1-2 also reduced bFGF- or VEGF-induced phosphorylation of FAK at Y397. Interestingly, we noticed phosphorylation of FAK at Y397 at low levels in the cells treated with TK1-2 alone in the absence of growth factor (Fig. 2A and B). Therefore, we tested whether the phosphorylation levels of FAK and ERK are dependent on the amount of TK1-2 without growth factor stimulation. FAK phosphorylation was increased on TK1-2 treatment, although the increased levels are low (Fig. 2C). Such FAK activation has also been reported in the case of angiostatin (35). However, ERK1/2 phosphorylation by TK1-2 was detected at very low levels compared with bFGF-stimulated phosphorylation level. Therefore, we concluded that TK1-2 alone activates FAK at a low level, whereas TK1-2 markedly inhibits growth factor–induced FAK activation.

### TK1-2 Reduces Formation of Actin Stress Fibers and Focal Adhesions

Next, we investigated whether TK1-2 affects the cytoskeleton rearrangement in HUVEC. After bFGF stimulation for 30 min, actin stress fibers and focal adhesions were visualized with TRITC-phalloidin or anti-vinculin antibody, respectively. In the absence of TK1-2, bFGF-stimulated cells showed formation of many actin stress fibers crossed in the cytoplasm, and vinculin staining was visible like a short spike in the cytoplasm (Fig. 3). However, when HUVEC were pretreated with TK1-2 before bFGF stimulation, such treatment prevented formation of actin stress fibers and focal adhesions (Fig. 3; Supplementary Fig. S1).[1] The similar inhibition results were obtained in VEGF-induced cells (data not shown). From the results, it was suggested that TK1-2 effectively inhibits growth factor–induced formation of actin stress fibers and focal adhesions.

### Anti-$\alpha_2\beta_1$ Integrin Antibody Potently Inhibits Endothelial Cell Binding to Immobilized TK1-2

FAK integrates growth factor and integrin signals to promote cell migration, and alterations of activity of FAK are associated with engagement of integrin receptors (34, 36). Because TK1-2 did not only inhibit growth factor–induced FAK activation and formation of actin stress fibers and focal adhesions but also itself activated FAK at a low level, we hypothesized that antimigratory activity of TK1-2 may be mediated by vascular integrins. To test this idea, we examined whether TK1-2 binds to endothelial cells and its binding involves integrins. First, when we carried out cell adhesion assay, we found that adhesion of HUVEC to TK1-2-coated dishes was increased in a dose-dependent manner at similar levels to recombinant angiostatin kringle 1-3 (PK1-3; Fig. 4A). When F-actin of attached cells was visualized by immunofluorescence, the cells adhered to immobilized TK1-2 did not form apparent actin stress fibers compared with the cells attached on fibronectin, but they were more spread out than the cells attached on BSA that remained round shaped (Fig. 4A, *bottom*; Supplementary Fig. S2).[1]

[1] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Because HUVEC adhere and spread onto immobilized TK1-2, we examined whether integrin blocking antibodies affect HUVEC binding to immobilized TK1-2. The integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ are up-regulated during tumor angiogenesis (37), and HUVEC express integrin subunits $\beta_1$, $\alpha_2$, $\alpha_5$, and $\alpha_v$ (38). Therefore, we chose three blocking antibodies against integrin $\alpha_2\beta_1$, $\alpha_v\beta_3$, and $\alpha_5\beta_1$ for the test. Interestingly, anti-$\alpha_v\beta_3$ or anti-$\alpha_5\beta_1$ antibody inhibited cellular attachment to TK1-2 at a certain level, whereas anti-$\alpha_2\beta_1$ antibody drastically reduced it (Fig. 4B). Under identical adhesion experiment conditions, pretreatment of soluble TK1-2 also inhibited the cellular attachment to immobilized TK1-2, suggesting specificity of HUVEC binding to TK1-2. Therefore, the results suggest that TK1-2 may interact with HUVEC primarily through integrin $\alpha_2\beta_1$ and, to a certain extent, through the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins.

### Interaction of TK1-2 with Integrin $\alpha_2\beta_1$ Is Involved in Antimigratory Effect of TK1-2

Because TK1-2 was suggested to predominantly interact with integrin $\alpha_2\beta_1$, we considered a possibility that antimigratory effect of TK1-2 is associated with TK1-2 binding to integrin $\alpha_2\beta_1$. Thus, we examined how a blocking antibody against integrin $\alpha_2\beta_1$, $\alpha_v\beta_3$, or $\alpha_5\beta_1$ affects the antimigratory effect of TK1-2. In the preliminary experiments, we examined the concentrations of integrin blocking antibodies to result in reduction of ~20% to 30% of VEGF-induced migration. Soluble anti-$\alpha_2\beta_1$ antibody inhibited VEGF-induced migration of HUVEC in a dose-dependent manner, and at a concentration of 1 μg/mL, it inhibited migration of HUVEC by ~30% (data not shown). Anti-$\alpha_v\beta_3$ and anti-$\alpha_5\beta_1$ blocking antibodies exerted ~30% inhibition of migration at 0.05 and 5 μg/mL, respectively. When cells were pretreated with anti-$\alpha_2\beta_1$ antibody at 1 μg/mL before TK1-2 treatment, anti-$\alpha_2\beta_1$ antibody was able to prevent the inhibition of migration by TK1-2 (Fig. 5A). On the contrary, anti-$\alpha_v\beta_3$ or anti-$\alpha_5\beta_1$ antibody could not rescue TK1-2-inhibited migration. Collectively, these data support that inhibition of endothelial cell migration by TK1-2 is mediated primarily by integrin $\alpha_2\beta_1$.

Next, we examined whether TK1-2 could bind to integrin $\alpha_2\beta_1$. We compared the binding level of each monoclonal integrin antibody between untreated and TK1-2-treated cells after TK1-2 treatment by fluorescence-activated cell sorting analysis to assess the level of integrins bound to TK1-2, which would be inaccessible to anti-integrin antibodies. Indeed, pretreatment of TK1-2 significantly reduced the binding level of anti-$\alpha_2\beta_1$ integrin antibody to HUVEC, whereas slight alteration in binding by anti-$\alpha_v\beta_3$ or anti-$\alpha_5\beta_1$ $\alpha_5\beta_1$ integrin antibody was observed on TK1-2 treatment (Fig. 5B). Therefore, this result suggests that TK1-2 interacts primarily with integrin $\alpha_2\beta_1$.

### Down-Regulation of $\alpha_2$ Integrin Prevents the Antimigratory Effect of TK1-2

To confirm the role of $\alpha_2\beta_1$ integrin in mediating the inhibitory effect of TK1-2 on HUVEC migration, we employed the $\alpha_2$ integrin knockdown system. As shown in Fig. 6A, $\alpha_2$-siRNA-transfected HUVEC showed marked reduction of $\alpha_2$ integrin expression compared with control scrambled siRNA transfectant, LipofectAMINE alone transfectant, or nontransfected HUVEC. $\alpha_2$-siRNA-transfected cells showed reduction of the overall migration response to VEGF (to 30-40% of nontransfected cells). It might be due to down-regulation of $\alpha_2$ integrin. However, VEGF-induced migration of $\alpha_2$-siRNA transfectants was not inhibited by TK1-2, whereas three other control cells showed similar inhibition of migration by TK1-2 (Fig. 6B). On the contrary, plasminogen kringle 5, to which HUVEC binding was not inhibited by anti-integrin $\alpha_2\beta_1$ antibody (data not shown), markedly inhibited VEGF-induced migration of $\alpha_2$-siRNA

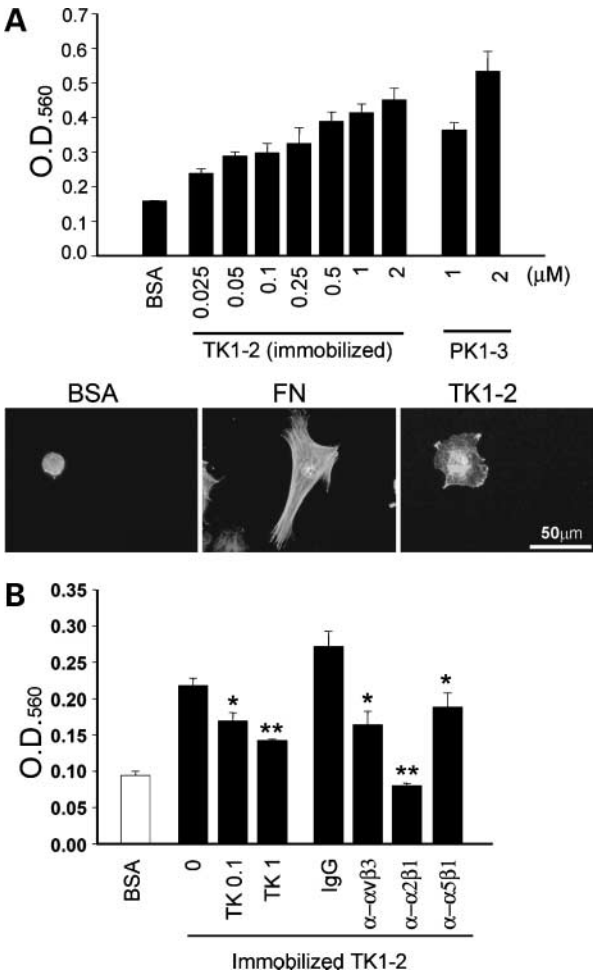


**Figure 4.** Adhesion of endothelial cells to immobilized TK1-2 and its blockage by anti-$\alpha_2\beta_1$ integrin antibody. **A,** HUVEC were seeded onto 96-well plates or coverslips coated with the indicated concentration of TK1-2, PK1-3, BSA, or fibronectin (*FN*) and incubated for 90 min. The attached cells were stained with crystal violet, and the stained dye was dissolved followed by measurement of absorbance at 560 nm. Mean ± SE. F-actin of the attached cells on coated coverslips was visualized by staining the cells with TRITC-conjugated phalloidin and 4′,6-diamidino-2-phenylindole (*bottom*). **B,** HUVEC were incubated with an anti-integrin antibody (10 μg/mL), control IgG (10 μg/mL), or soluble TK1-2 (0.1 and 1 μmol/L) for 30 min and then plated onto TK1-2-coated plates (2 μmol/L). The attached cells were assessed by the same method. *, $P < 0.05$; **, $P < 0.005$, compared with relevant control.

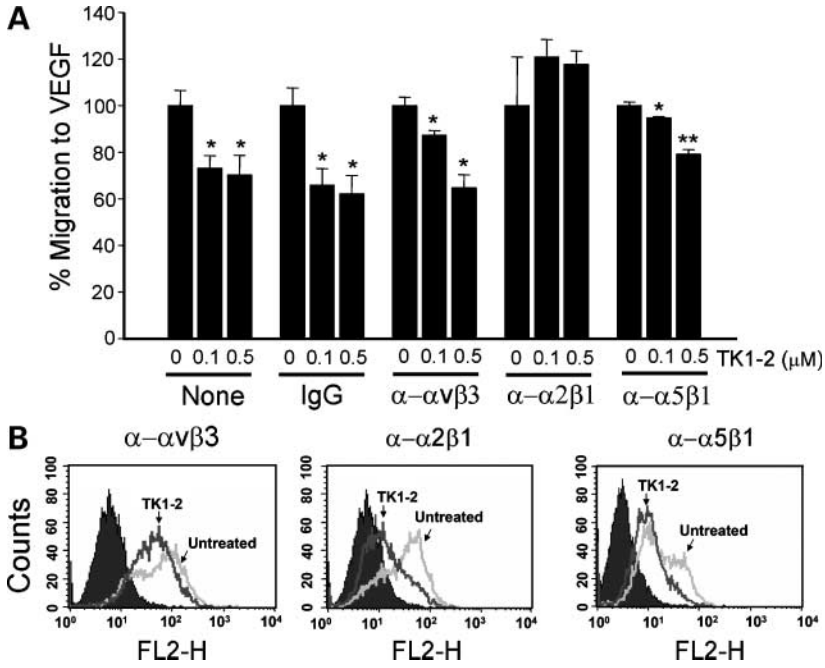


**Figure 5.** Antimigratory activity of TK1-2 is prevented by a blocking antibody against integrin $\alpha_2\beta_1$. **A,** HUVEC were pretreated with the anti-$\alpha_2\beta_1$, anti-$\alpha_v\beta_3$, or anti-$\alpha_5\beta_1$ integrin antibody for 30 min and then treated with TK1-2 for another 30 min. The cells were added to the top of each migration chamber and allowed to migrate in the presence of VEGF (2 ng/mL) to the underside of the chamber for 5 h. Cell Migration is presented as relative percentage compared with relevant control induced by VEGF. *, $P < 0.05$; **, $P < 0.005$, compared with relevant VEGF alone treatment control. **B,** HUVEC were treated with 1 μmol/L TK1-2 for 30 min and then incubated with the indicated anti-integrin antibody (10 μg/mL) for 30 min followed by incubation with the Cy3-conjugated secondary antibody. The cells were washed, fixed, and analyzed by flow cytometry.

transfectants (Fig. 6B). We also confirmed the binding level of anti-$\alpha_2\beta_1$ integrin antibody between the transfectants upon pretreatment of TK1-2 by detecting that $\alpha_2$-siRNA transfectants showed no change in anti-$\alpha_2\beta_1$ antibody binding upon TK1-2 treatment in contrast to control scrambled siRNA transfectants (Fig. 6C). Therefore, these data strongly support that TK1-2 inhibits endothelial cell migration through $\alpha_2\beta_1$ integrin.

## Discussion

TK1-2 has been identified as a novel angiogenesis inhibitor through *in vitro* and *in vivo* studies (5–7). However, its molecular targets and mechanism of action remain to be elucidated. In the present study, we showed $\alpha_2\beta_1$ integrin as a putative molecular target of TK1-2 for antiangiogenesis through examining its mechanism of action in inhibition of endothelial cell migration.

Integrin $\alpha_2\beta_1$ has been implicated in angiogenesis. VEGF induces $\alpha_1\beta_1$ and $\alpha_2\beta_1$ expression by microvascular endothelial cells, and $\alpha_1\beta_1$ and $\alpha_2\beta_1$ antibody antagonists inhibit VEGF-driven angiogenesis *in vivo* (39). *In vitro*, microvascular endothelial cell attachment through $\alpha_2\beta_1$ supports robust VEGF activation of ERK1/2, and antagonism of $\alpha_2\beta_1$ integrin suppresses dermal microvascular chemotaxis (25). In our point of view, interaction of TK1-2 with integrin $\alpha_2\beta_1$ as a partial agonist, or an antagonist, may provide an important mechanism of action of TK1-2 for inhibiting growth factor–induced ERK1/2 activation and cytoskeleton rearrangement and consequently for inhibition of cell migration and angiogenesis. Because TK1-2 itself activates FAK at a low level, TK1-2 may act as a partial agonist to elicit the same effect as antagonistic effect. The notion that $\alpha_2\beta_1$ integrin mediates the potent antiangiogenic and antitumor activity of angiogenesis inhibitors has also been proven in other angiogenesis inhibitors such as endorepellin and angiocidin (40, 41).

Interestingly, in contrast to endothelial cells undergoing collagen I–induced capillary morphogenesis mediated by $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (42), fibroblasts, which also express integrin $\alpha_2\beta_1$, do not respond to collagen I with increased actin polymerization, changes in cell shape, or cellular alignment into cords; this may relate to the fact that fibroblasts normally reside within interstitial collagens. On the other hand, endothelial cells encounter collagen I only during the sprouting and invasive stages of angiogenesis, thereby indicating that collagen I is appropriately situated to serve as a stimulus for angiogenesis and that integrin $\alpha_2\beta_1$ can be an important mediator. In fact, TK1-2 inhibited the migration of endothelial cells but not the migration of cancer cells (HT1080, U87, and A549) at the identical concentration range, with no relationship with $\alpha_2\beta_1$ expression (data not shown). Thus, it could be explained in part by the mechanism of action of TK1-2 mediated by integrin $\alpha_2\beta_1$.

Although $\alpha_2\beta_1$ is a dominant receptor for TK1-2, we still could not exclude the binding ability of TK1-2 to other integrins or receptors for explaining its mechanism of action. Inhibition of cellular binding to immobilized TK1-2 by a blocking antibody against integrin $\alpha_v\beta_3$ or $\alpha_5\beta_1$ was observed at a low level with significance, although inhibition of migration by TK1-2 was not completely prevented by the pretreatment of anti-$\alpha_v\beta_3$ or anti-$\alpha_5\beta_1$ integrin antibody. Therefore, there still will be a possibility of additive effects mediated by integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in the antiangiogenic activity of TK1-2 albeit at a different level of contribution. In the case of angiostatin, it has been reported to bind to $\alpha_v\beta_3$ integrin (19). In their study, integrin $\alpha_2\beta_1$ was not examined for interaction with

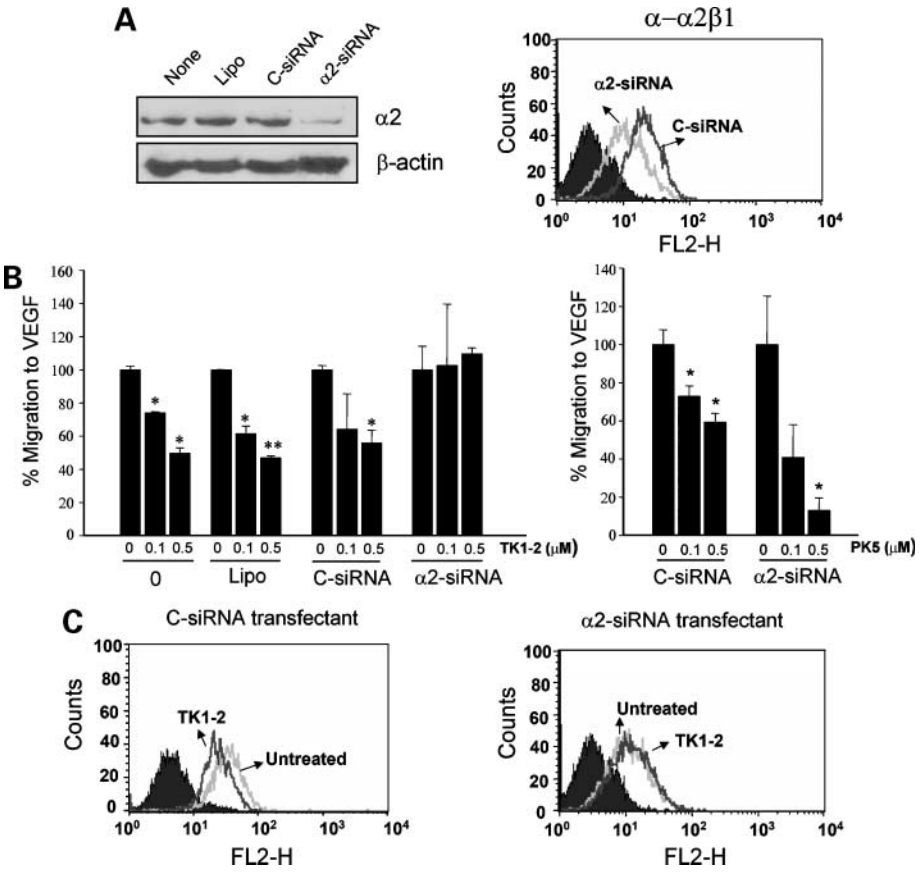


**Figure 6.** Antimigratory effect of TK1-2 is integrin $\alpha_2$ dependent. **A,** HUVEC were transfected with $\alpha_2$-siRNA, scrambled siRNA (*C-siRNA*), or LipofectAMINE alone for 48 h. Then, knockdown of $\alpha_2$ integrin was confirmed by Western blot analysis and fluorescence-activated cell sorting analysis. **B,** after transfection for 48 h, the cells were subjected to a modified Boyden chamber migration assay after pretreatment of TK1-2 or plasminogen kringle 5 for 30 min. *, $P < 0.05$; **, $P < 0.005$, compared with relevant VEGF alone treatment control. **C,** transfectants were treated with 1 μmol/L TK1-2 for 30 min and then incubated with the anti-$\alpha_2\beta_1$ integrin antibody (10 μg/mL) for 30 min followed by incubation with the Cy3-conjugated secondary antibody. The cells were washed, fixed, and analyzed by flow cytometry.

angiostatin, although anti-$\beta_1$ integrin antibody had a marginal effect. When we examined the cellular binding of angiostatin in our hands, we found that HUVEC binding to angiostatin was inhibited not only by anti-$\alpha_v\beta_3$ antibody but also by anti-$\alpha_2\beta_1$ antibody albeit at a lower level than cellular binding to TK1-2 (data not shown). Consistent with integrin bindings, angiostatin also diminishes growth factor–induced ERK phosphorylation (43) and causes FAK activation (35). Apolipoprotein(a) kringle domain has been also shown to inhibit ERK1/2 activation previously (32). Therefore, it raises an intriguing question whether antiangiogenic activities of other kringles are also mediated by integrins with a similar repertoire.

While we narrowed down a major receptor for TK1-2 as $\alpha_2\beta_1$ integrin, we found that the major recognition site of collagen type I for $\alpha_2\beta_1$ integrin, DGEA, is similar to DGDA sequence within TK1-2 (44). DGEA peptide inhibits collagen-induced platelet reactivity as an antagonist through $\alpha_2\beta_1$ integrin (45) and also inhibits endothelial progenitor cell differentiation on collagen matrix (46). Interestingly, such similar sequence, DGDA, exists only in kringle 2 of tissue-type plasminogen activator among kringle molecules, which is consistent with the result that the kringle domain 2 alone is a novel molecule for antiangiogenic therapy (8). Therefore, a possibility of DGDA sequence associating with antimigratory effect of TK1-2 has been raised, and we are studying the question whether TK1-2 perturbs $\alpha_2\beta_1$ integrin through DGDA sequence and whether DGDA is able to function as an antiangiogenic peptide. At present, we obtained the data supporting this idea.[2]

In summary, our data suggest that TK1-2 inhibits growth factor–induced ERK/FAK activation, formation of stress fibers and focal adhesions, and endothelial cell migration and that its inhibitory activity is mediated in part by integrin $\alpha_2\beta_1$. This study also emphasizes $\alpha_2\beta_1$ integrin as an effective target for antiangiogenic activity and provides also some insights on the integrin-mediated mechanism of action of kringle-based angiogenesis inhibitors.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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