FAS1-domain protein inhibits VEGF165-induced angiogenesis by targeting the interaction between VEGFR-2 and αvβ3 integrin

(Running title: The angiostatic activity of FAS1 domain protein)

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Abstract

It is known that vascular endothelial growth factor receptors (VEGFRs) and integrins interact with each other to regulate angiogenesis. We reported previously that the fasciclin 1 (FAS1) domain-containing protein, TGFBIp/βig-h3 (transforming growth factor-β–induced protein) is an angiogenesis regulator that inhibits both endothelial cell migration and growth via αvβ3 integrin. In an attempt to target the interaction between VEGFR-2 and αvβ3 integrin, we determined whether the FAS1-domain region of TGFBIp/βig-h3 (FAS1-domain protein) can block the interaction between the two receptors, leading to the suppression of angiogenesis. In this study, we demonstrated that FAS1-domain protein inhibits VEGF165-induced endothelial cell proliferation and migration via αvβ3 integrin, resulting in the inhibition of VEGF165-induced angiogenesis. We also defined a molecular mechanism by which FAS1-domain protein blocks the association between αvβ3 integrin and VEGFR-2, showing that it binds to αvβ3 integrin but not to VEGFR-2. Blocking the association of these major angiogenic receptors with FAS1-domain protein inhibits signaling pathways downstream of VEGFR-2. Collectively, our results indicate that FAS1-domain protein, in addition to its inhibitory effect on αvβ3 integrin-mediated angiogenesis, also inhibits VEGF165-induced angiogenesis. Thus, FAS1-domain protein can be further developed into a potent anticancer drug that targets two principal angiogenic pathways.

Key words: FAS1 domain protein, Tumor angiogenesis, αvβ3 integrin, and VEGFR-2
Introduction

TGFβIp/βig-h3 is an extracellular matrix protein that is induced by transforming growth factor-β (TGF-β) in a variety of cell types (1-4). The TGFβIp/βig-h3 protein contains a C-terminal arginyl-glycyl-aspartic acid (RGD) sequence and four homologous internal repeat domains similar to the Drosophila protein fasciclin-1 (FAS1) (5). We reported that C-terminal cleavage of TGFβIp/βig-h3 yields RGD-containing peptides that, in turn, induce apoptosis in several cell lines (6), and have an impact on a variety of biological and pathological processes (7). Recent studies have shown that the amyloidogenic region of the FAS1-domain of TGFβIp/βig-h3, produced through proteolytic processing by the chymotrypsin-like serine protease HtrA1, encompasses the Y571-R588 peptide (8). The fourth FAS1-domain region of TGFβIp/βig-h3 (hereafter referred to as FAS1-domain protein) mediates cell adhesion and migration via interaction with integrins (9, 10). We reported previously that FAS1-domain protein mediates endothelial cell adhesion and migration via αvβ3 integrin, which acts as an anti-angiogenic regulator. FAS1-domain protein can inhibit both endothelial cell migration and growth by blocking two signal cascades—the AKT/mTOR (mechanistic target of rapamycin) and Raf/ERK (extracellular signal-regulated kinase) pathways—both of which are initiated by the activation of αvβ3 integrin (11).

Vascular endothelial growth factor A (VEGFA) is one of the most relevant factors in tumor angiogenesis. Commonly referred to as VEGF, it exists in four different isoforms (composed of 121, 165, 189, and 206 amino acids in humans), which are generated by alternative splicing of a single pre-mRNA (12). VEGF165 is the predominant isoform and is commonly overexpressed in a variety of tumors. Two structurally related receptors for VEGF, VEGFR-1 and VEGFR-2, have been identified, and their expression has been shown to predominate in endothelial cells (13). In particular, VEGFR-2 plays a critical role in
mediating the actions of VEGFs, including mitogenic, chemotactic, and vascular permeabilizing effects on the vasculature (14-17).

Various studies have suggested that integrins and their cognate matrix protein ligands collaborate closely with growth factors in transducing signals through the phosphoinositide 3-kinase (PI3K)-AKT pathway (18-20). The interaction between integrin and growth factor receptors is bidirectional such that many of the integrin-induced signaling pathways can be activated through binding of soluble growth factors to their receptors (21). In a similar fashion, many growth factor receptors are also activated by the interaction of integrins with their extracellular matrix ligands (22-26). Indeed, vitronectin, a natural ligand for \( \alpha v \beta 3 \), enhances VEGFR-2 phosphorylation (23, 27). Interestingly, several growth factor receptors, including platelet-derived growth factor receptor (PDGFR)-\( \beta \), interferon-like growth factor receptor (IGFR), epidermal growth factor receptor (EGFR) and VEGFR-2, associate with activated \( \alpha v \beta 3 \) integrin (24, 28-31). Blocking \( \beta 3 \), but not \( \beta 1 \), inhibits VEGF-induced VEGFR-2 phosphorylation, leading to drastically reduced cellular migration and proliferation (23, 32, 33).

Although many attempts have been made to target either \( \alpha v \beta 3 \) integrin or VEGFR-2, only minimal advances have been made in targeting the interaction between the VEGF receptor and \( \alpha v \beta 3 \) integrin as a strategy for developing anti-angiogenic therapies. The primary objective of this study was to determine whether FAS1-domain protein, which acts as an \( \alpha v \beta 3 \) integrin blocker, can regulate angiogenesis by inhibiting the interaction between the two major angiogenic pathways—the VEGF\(_{165}\)-mediated angiogenic pathway and the \( \alpha v \beta 3 \) integrin-mediated pathway. In this study, we show that FAS1-domain protein inhibits VEGF\(_{165}\)-induced angiogenesis by blocking the association between VEGFR-2 and \( \alpha v \beta 3 \) integrin. FAS1-domain protein-induced blockade of this association inhibits signaling
cascades downstream of VEGFR-2. These findings suggest that FAS1-domain protein, a soluble αvβ3 integrin binding molecule, effectively functions as an inhibitor of tumor angiogenesis by blocking both αvβ3 integrin- and VEGF-mediated angiogenic pathways.
Materials and methods

DNA construction and purification

The bacterial expression vector for the fourth FAS1-domain protein of the human TGFBIp/βig-h3 has been previously described (11). Briefly, the recombinant protein was purified using a nickel-nitrilotriacetic acid-agarose column (Qiagen) according to the manufacturer’s instruction. Endotoxin was removed by using polymixin B agarose (Pierce, Rockford, IL).

Cell transfection and infection

Primary human umbilical vein endothelial cells (HUVEC), β3/HEK293, and β5/HEK293 were cultured as previously described (11). In brief, HUVECs were cultured in M199 medium (Sigma Chemical Co., Louis, MO) supplemented with 20% fetal bovine serum and 100 units/ml of penicillin-streptomycin for no more than 5 passages. HEK293 (Human embryonic kidney) and HUVEC cells transfected with a human β3 integrin-expressing vector or a human β5 integrin-expressing vector were kindly provided by Dr. Jeffrey Smith (Burnham Institute, San Diego). β3/HEK293 and β5/HEK293 were cultured in DMEM medium (Gibco BRL., Gaithersburg, MD) containing high glucose with 10% fetal bovine serum and 100 units/ml of penicillin-streptomycin. HUVEC, β3/HEK293, and β5/HEK293 cells were transfected with murine VEGFR-2 DNA using Lipofectamine (Invitrogen, Branford, CT) in accordance with the manufacturer’s protocols. In brief, β3/HEK293 and β5/HEK293 cells were grown to a 50-60% confluency in 10-cm plates prior to the introduction of a plasmid (4-6 μg) containing the murine VEGFR-2 or the control vector. At 40 to 48 h after transfection, the cells were collected and utilized in subsequent functional studies. HUVEC cells were infected with Lentiviral Particles containing with small interfering hairpin RNAs (shRNA) against β3 integrin and a scrambled control shRNA (Santa
Cruze Biotechnology, Inc.) in accordance with the manufacturer’s protocols. In brief, HUVEC cells were grown to approximately 50% confluent on the day of infection. HUVEC cells were removed media and incubated with polybrene (10 mg/ml) prior to the infection of Lentiviral Particles. At 48 h after infection, the clones expressing the scrambled control shRNA and β3 integrin shRNA were selected by puromycin (2 μg/ml) for 5 days and utilized in functional studies.

**In vitro and in vivo angiogenesis assays**

An in vitro endothelial tube formation was conducted as previously described (11). Matrigel (BD Bioscience, San Jose, CA) was added (100 μl) to a 96-well plate and permitted to polymerize. The cells were suspended in serum-free medium at a density of $3 \times 10^5$ cells/ml, and 0.1 ml of the cell suspension was preincubated for 30 min with the indicated FAS1-domain protein concentrations and then stimulated with 50 ng/ml of VEGF$_{165}$. The cell suspension was then added to each Matrigel-coated well. The cells were incubated for 6 to 8 h at 37 °C. The cells were then imaged, and branch points from 4 to 6 high-power fields ($\times$ 200) were counted and averaged. Each group was evaluated in triplicate. Then, an in vivo Matrigel plug assays were conducted as previously described (11). In brief, 5- to 6-week-old male C57BL/6 mice were used. Matrigel (BD Bioscience) was mixed with 20 units/ml of heparin and 0.25 μg/ml of VEGF$_{165}$ with or without the indicated concentration of the FAS1-domain protein. The Matrigel mixture (500 μl) was injected subcutaneously into the mouse flanks and the mice were sacrificed after 9 days, after which the Matrigel plugs were removed and fixed in 4% paraformaldehyde. The plugs were then paraffin-embedded, sectioned, and stained with anti-CD31 antibody (BD Bioscience). Sections were examined via fluorescence microscopy, and the number of CD31 positive blood vessels from 4 high-power fields ($\times$ 200) were counted and averaged. Each group consisted of five or six Matrigel plugs.
Proliferation assay

The cells were seeded at a density of 3,000 cells per well into 96-well plates and incubated overnight. The next day, the media was removed, and the cells were washed twice with PBS and starved for 24 h in serum-free medium containing 0.1% bovine serum albumin (BSA). The following day, the FAS1-domain protein was added to the cells 30 min prior to stimulation with 20 ng/ml of VEGF_{165} in serum-free media. After 48 h of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical, St. Louis, MO) was added to each well and lysed with DMSO, then quantified via the measurement of $A_{570\ nm}$ using an ELISA plate reader (Bio-Rad, Hercules, CA). Each treatment was conducted in triplicate, and the entire experiment was performed at least three times independently.

Migration assay

The inhibitory effect of the FAS1-domain protein on VEGF_{165}-induced chemotaxis was assessed using human umbilical vein endothelial cells (HUVECs) on transwell plates (8 μm pore size, Costar, Cambridge, MA). The undersurface of the membrane was coated with 2% BSA, 5 μg/ml collagen type I or vitronectin (Promega, Madison, WI) at 4 °C and blocked for 1 h at room temperature with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA). HUVECs were grown overnight in M199 medium (Sigma Chemical Co.) containing 20% FBS. Following trypsinization, 30,000 cells were diluted in M199 medium containing 0.5% FBS and seeded on the upper chamber wells, together with or without the indicated FAS1-domain protein concentrations. M199 medium containing 0.5% FBS plus 20 ng/ml of VEGF_{165} was placed in the lower chamber wells as a chemoattractant. The chamber was incubated for 6 h at 37 °C. Migration was terminated via the removal of the cells from the upper compartment of the filter using a cotton swab, and the filters were fixed with 8% glutaraldehyde and stained with crystal violet. The extent of cell migration within each well
was evaluated via light microscopy and cells were counted in five randomly selected microscopic high-power fields.

The inhibitory effects of the FAS1-domain protein on VEGF165-induced chemotaxis were assessed on β3/HEK293 and β5/HEK293 using transwell plates (8 μm pore size, Costar, Cambridge, MA). The undersurface of the membrane was coated with 5 μg/ml of collagen type I at 4 °C, then blocked for 1 h at room temperature with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA). The cells were grown overnight in DMEM medium containing 10% FBS. After trypsinization, 30,000 cells were diluted in DMEM medium containing 0.5% FBS and seeded on the upper chamber wells, with or without the indicated concentration of the FAS1-domain protein. DMEM containing 0.5% FBS plus 20 ng/ml of VEGF165 was placed in the lower chamber wells as a chemoattractant. The chamber was then incubated for 6 h at 37 °C. The migrated cells were then quantified as described above.

Cell cytotoxicity assay

The HUVECs were suspended in M199 medium containing 0.5% FBS at a density of $1 \times 10^6$ cells/ml, and 0.1 ml of the cell suspension was treated for 1 h and 6 h at 37 °C with the indicated FAS1-domain protein concentrations. The percentage of live cells was determined using a Luna™ automated cell counter (Logos Biosystems, Inc.) with 0.4% trypan blue staining.

Immunoprecipitation and immunoblotting

At 48 h after transfection, the cells were rendered quiescent via 24 h of starvation in serum-free medium containing 0.1% bovine serum albumin (BSA). The cells were pre-treated for 30 min with 10 μM FAS1-domain protein prior to exposure to 20 ng/ml of VEGF165 for 5 min.
The cells were then lysed in a cell lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF and 10% glycerol). After determining the protein concentration via the Bradford method (Bio-Rad, Hercules, CA), 100 μg of protein from each of the sample was subjected to SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden), which were blocked overnight in TBS-T (50 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20) containing 5% non-fat dry milk. The membranes were then incubated for 16 h with primary antibodies at 4 °C and washed three times in TBS-T. The proteins were visualized using enhanced chemiluminescence reagent (Pierce, Rockford, IL) and exposed to film. The antibodies utilized were: anti-β3, anti-β5, anti-phospho-serine 473 AKT, anti-phospho-tyrosine 204 ERK, and anti-VEGFR-2 antibodies (Santa Cruz Biotechnology), anti-phospho-tyrosine 1175 VEGFR-2, anti-phospho-tyrosine 182 P38 MAPK, anti-AKT, and anti-P38 MAPK antibodies (Cell signaling Technology, Inc.), anti-β-actin (Sigma-Aldrich), and anti-ERK antibody (BD Transduction Laboratories, Lexington, KY). For the co-immunoprecipitation assay, 1 mg of each protein was pre-cleared via incubation for 1 h with protein A-Sepharose beads (Amersham Pharmacia Biotech). The samples were then incubated for 12 h with anti-VEGFR-2 antibody or anti-β3 antibody (Chemicon) at 4 °C, and the immune complexes were recovered on protein A-Sepharose beads. The immunoprecipitates were washed four times with lysis buffer, twice with the same buffer without Triton X-100, and once with PBS. After boiling, the supernatants were separated via SDS-PAGE, transferred to nitrocellulose membranes, and probed with primary anti-phospho-tyrosine antibody (PY20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-β3 antibody (Cell Signaling Technology, Inc.) and anti-VEGFR-2 antibody. The proteins were visualized as described above. Relative protein expression levels were measured by
densitometry using Image J (version 1.34s).

**Surface plasmon resonance (SPR) for cell-protein interaction**

Experiments were performed with a Reichert SR7500DC (Reichert, Inc.). Initially, mSAM 10% COOH surface was activated using 175 μl of 50 mM N-hydroxysuccinimide (NHS) and 200 mM N-ethyl-N’-(3-di-ethylaminopropyl) carbodiimide (EDC) at a flow rate of 25 μl/min for 7 min. The activated surface was coated with 50 μg/ml of poly-L-Lysine in 10 mM sodium acetate buffer (pH 4.0), followed by a blocking step using 1M ethanolamine hydrochloride at a flow rate of 25 μl/min for 8 min. Suspended cells (400,000 cells/ml) were injected at a flow rate of 25 μl/min for 10 min. HEK293 cells, which were used as a control surface were passed over the right channel and β3/HEK293 or VEGFR-2/HEK293 cells were passed over the left channel, respectively. PBS (None) or 7.8 μM FAS1-domain protein was then injected at a flow rate of 25 μl/min for 7 min. Differential sensorgrams from the transfected cell channel and mock cell channel were directly recorded, and utilized for kinetic analysis. Specific binding of the FAS1-domain protein to β3/HEK293 or VEGFR-2/HEK293 cells was subtracted from binding of PBS to both cells, respectively. The Reichert Autolink SPR-software was used for data collection, and the Biologic Scrubber 2 software was used for kinetic data analysis.

**Statistical analysis**

All values are expressed as the means ± SE. The statistical significance between the experimental and control groups was evaluated via ANOVA test. A p-value of < 0.05 was considered to be statistically significant, and is indicated by an asterisk over the value.
Results

FAS1-domain protein inhibits VEGF$_{165}$-induced angiogenesis *in vitro* and *in vivo*.

To investigate the potential of FAS1-domain protein to inhibit VEGF$_{165}$-induced angiogenesis *in vitro*, we assessed its ability to disrupt VEGF$_{165}$-induced endothelial cell tube formation in Matrigel. VEGF$_{165}$-stimulated HUVEC cells cultured on Matrigel aligned rapidly and formed hollow tube-like structures, as shown in Figure 1A. FAS1-domain protein inhibited VEGF$_{165}$-induced tube formation in a dose-dependent manner; cells treated with 10 μM FAS1-domain protein appeared scattered, similar to the appearance of cells cultured in the absence of VEGF$_{165}$ (Fig. 1A, B). We then verified the ability of FAS1-domain protein to inhibit VEGF$_{165}$-induced angiogenesis *in vivo*. For this purpose, Matrigel plugs containing VEGF$_{165}$, with or without recombinant FAS1-domain protein, were subcutaneously injected into C57BL/6 mice. Nine days after implantation, we evaluated the extent of blood vessel invasion into the Matrigel plugs. Similar to the results obtained in tube-formation assays, VEGF$_{165}$-induced blood vessel invasion was inhibited by FAS1-domain protein in a dose-dependent manner (Fig. 1C, D).

FAS1-domain protein inhibits VEGF$_{165}$-induced cell proliferation and migration via αvβ3 integrin.

In order to understand how FAS1-domain protein inhibits VEGF-induced angiogenesis, we first determined whether it inhibited the VEGF$_{165}$-stimulated growth of HUVEC cells. Cells were preincubated with FAS1-domain protein and then stimulated with VEGF$_{165}$ in serum-free medium. After 48 h of incubation, FAS1-domain protein inhibited the VEGF$_{165}$-stimulated growth of HUVEC cells in a dose-dependent manner (Fig. 2A). In order to determine whether αvβ3 integrin is involved in FAS1-domain protein-mediated inhibition of VEGF$_{165}$-stimulated growth, we utilized HEK293 cells stably transfected with a human β3 or...
β5 integrin expression vector. β3- or β5-integrin–positive cells were then selected by sorting with antibodies against αvβ3 (P112-4C1) or αvβ5 integrin (P1F6) (34). HEK293 cells do not express endogenous β3, β5 or VEGFR-2; thus, VEGF165 does not stimulate HEK293 cell proliferation (data not shown). These cells were additionally transiently co-transfected with a VEGFR-2 expression vector. In VEGFR-2/β3/HEK293 cells, FAS1-domain protein inhibited VEGF165-stimulated growth in a dose-dependent manner, an effect not observed in VEGFR-2/β5/HEK293 cells (Fig. 2B). Next, we assessed the ability of FAS1-domain protein to inhibit VEGF165-induced endothelial cell migration using transwell plates whose undersurface was coated with either collagen type I or vitronectin. The extent of endothelial migration was greater for vitronectin-coated surfaces than for collagen-coated surfaces, but VEGF165 treatment enhanced migration on both surfaces. Addition of FAS1-domain protein (20 μM) markedly reduced the number of HUVEC cells migrating in response to VEGF165 stimulation not only on the vitronectin-coated surface but also on the collagen type I-coated surfaces (Fig. 2C, D). An evaluation of the percentage of live HUVEC cells after FAS1-domain protein treatment using a Luna automated cell counter showed that FAS1-domain protein did not exert a cytotoxic effect (Supplemental Fig. 1).

αvβ3 integrin is not a receptor for collagen type I, but FAS1-domain protein inhibited VEGF165-enhanced migration on collagen type I. As we reported previously, FAS1-domain protein interacts with αvβ3 integrin (11). Thus, we questioned whether FAS1-domain protein inhibited VEGF-induced cell migration on collagen type I in an αvβ3 integrin dependent-manner. Again utilizing VEGFR-2/β3/HEK293 and VEGFR-2/β5/HEK293 cells, we found that VEGF165 enhanced the migration of both cell types on collagen type I (Fig. 2E, F). Notably, the observed increase in migration was significantly inhibited by FAS1-domain protein in VEGFR-2/β3/HEK293 cells but not in VEGFR-2/β5/HEK293 cells, suggesting that
the inhibition of VEGF$_{165}$-mediated cell migration by FAS1-domain protein is dependent on αvβ3 integrin, regardless of substrate.

**FAS1-domain protein inhibits association of αvβ3 integrin with VEGFR-2 and attenuates VEGF$_{165}$-induced VEGFR-2 phosphorylation.**

Previous studies have shown that VEGF promotes the association of VEGFR-2 with αvβ3 integrin, which induce cell proliferation and migration in a synergistic manner (23). Using VEGFR-2/β3/HEK293 and VEGFR-2/β5/HEK293 cells, we tested whether FAS1-domain protein inhibited the association of αvβ3 integrin with VEGFR-2 upon VEGF$_{165}$ stimulation. VEGF$_{165}$ stimulation increased the association of αvβ3 integrin with VEGFR-2, tested using co-immunoprecipitation assays employing anti-VEGFR-2 antibody-bound protein A sepharose. Again, this increase was inhibited by FAS1-domain protein in VEGFR-2/β3/HEK293 cells (Fig. 3A, Supplemental Fig. 2A), but not in VEGFR-2/β5/HEK293 cells (data not shown). In control experiments, co-immunoprecipitation assays showed no binding of αvβ3 integrin to normal rabbit IgG-bound protein A-Sepharose following VEGF$_{165}$ stimulation, indicating that the observed interaction is specific (Supplemental Fig. 2B).

VEGFR-2 phosphorylation is essential to the mediation of VEGF-induced angiogenesis in the vasculature (17). Thus, we attempted to determine whether FAS1-domain protein inhibited VEGF$_{165}$-induced VEGFR-2 phosphorylation in HUVEC cells. To exclude the possibility of VEGFR-2 phosphorylation via αvβ3 integrin stimulation by substrate, we used collagen-coated plates. As shown in Figure 3B and Supplemental Figure 2C, VEGFR-2 in VEGF$_{165}$-stimulated HUVEC cells was phosphorylated to a significant degree, and this phosphorylation was inhibited by FAS1-domain protein. In order to determine whether αvβ3 integrin is responsible for the inhibitory activity of FAS1-domain protein on VEGF$_{165}$-induced VEGFR-2 phosphorylation, we utilized the over-expression system described above.
(VEGFR-2/β3/HEK293 and VEGFR-2/β5/HEK293 cells) as well as a knockdown system employing HUVEC cells transfected with small interfering hairpin RNAs (shRNA) against β3 integrin (β3 integrin shRNA/HUVEC cells) and a scrambled control shRNA (control shRNA/HUVEC cells). VEGF_{165}-stimulated VEGFR-2 phosphorylation was inhibited by FAS1-domain protein in VEGFR-2/β3/HEK293 and control shRNA/HUVEC cells but not in VEGFR-2/β5/HEK293 or β3 integrin shRNA/HUVEC cells, suggesting that FAS1-domain protein inhibits the phosphorylation of VEGFR-2 by blocking the association between αvβ3 integrin and the VEGF receptor (Fig. 3C-E and Supplemental Fig. 2D, E).

**FAS1-domain protein inhibits vitronectin-induced VEGFR-2 phosphorylation and association of αvβ3 integrin with VEGFR-2.**

In order to determine whether direct activation of integrin by extracellular matrix proteins is capable of triggering tyrosine phosphorylation of VEGFR-2, we plated serum-starved HUVEC and VEGFR-2/β3/HEK293 cells on vitronectin and measured VEGFR-2 phosphorylation by Western blotting. As shown in Figure 4 (A, B) and Supplemental Figure 3 (A, B), VEGFR-2 was profoundly phosphorylated in both HUVEC and VEGFR-2/β3/HEK293 cells plated on vitronectin, and the degree of phosphorylation was further enhanced by VEGF_{165}. However, VEGFR-2 was not phosphorylated in VEGFR-2/β3/HEK293 cells plated on collagen, which does not bind αvβ3 integrin (Supplemental Fig. 3C).

Next, we assessed the effect of VEGF_{165} on the ability of FAS1-domain protein to inhibit VEGFR-2 phosphorylation. FAS1-domain protein inhibited VEGFR-2 phosphorylation in both HUVEC and VEGFR-2/β3/HEK293 cells plated on vitronectin in the presence or absence of VEGF_{165}.

In order to determine whether activation of integrin by extracellular matrix proteins
enhanced the association of αvβ3 integrin with VEGFR-2, we plated serum-starved HUVEC and VEGFR-2/β3/HEK293 cells on vitronectin and subsequently tested cell lysates using co-immunoprecipitation assays, as described above. VEGFR-2 was associated with αvβ3 integrin in cells plated on vitronectin. Similar to its effects on VEGFR-2 phosphorylation, VEGF165 enhanced the association of VEGFR-2 with αvβ3 integrin in cells plated on vitronectin; this VEGF165-induced increase in association was inhibited by FAS1-domain protein in both HUVEC and VEGFR-2/β3/HEK293 cells (Fig. 4C, D and Supplemental Fig. 3D, E).

**FAS1-domain protein interacts with αvβ3 integrin but not with VEGFR-2.**

Because we had previously demonstrated that FAS1-domain protein interacts with αvβ3 integrin (11), we assumed that interference of the VEGFR-2–αvβ3 integrin interaction by FAS1-domain protein was due to the binding of FAS1-domain protein to αvβ3 integrin. We also examined whether FAS1-domain protein could interact directly with VEGFR-2 using surface plasmon resonance (SPR) analysis and recombinant FAS1-domain protein and VEGFR-2 extracellular domain. No direct interaction was observed in these experiments (data not shown). However, because recombinant β3 integrin and VEGFR-2 proteins may not adopt the same structure as the native proteins on the cell membrane, we conducted a cell-protein SPR assay using HEK293 cells transiently transfected with β3 integrin or VEGFR-2 (Fig. 5A, B). FAS1-domain protein bound to β3/HEK293 cells ($K_d, \sim 0.96 \pm 0.14 \mu M$) but not to VEGFR-2/HEK293 cells ($K_d, \text{ n.d.}$) (Fig. 5C, D), suggesting that FAS1-domain protein inhibits the association between αvβ3 integrin and VEGFR-2 by binding to αvβ3 integrin.

**VEGF165-induced signaling pathways are enhanced by activation of αvβ3 integrin and inhibited by FAS1-domain protein.**
Because we had determined that the activation of αvβ3 integrin enhanced VEGF<sub>165</sub>-stimulated VEGFR-2 phosphorylation and association with αvβ3 integrin, we attempted to confirm whether αvβ3 integrin activation could also enhance the VEGF<sub>165</sub>-stimulated phosphorylation of AKT, ERK, and P38 MAPK (mitogen-activated protein kinase) in VEGF<sub>165</sub>-stimulated VEGFR-2/β3/HEK293 cells plated on either vitronectin or collagen type I. As shown in Figure 6, each of these proteins was phosphorylated to a greater degree in VEGFR-2/β3/HEK293 cells plated on vitronectin than in those plated on collagen type I, and the phosphorylation of each molecule was inhibited by FAS1-domain protein. However, FAS1-domain protein did not inhibit phosphorylation of AKT, ERK, or P38 MAPK in VEGFR-2/β5/HEK293 cells plated on collagen type I (Supplemental Fig. 4).
Discussion

Angiogenesis is a multistep process in which new blood vessels are formed from preexisting vessels in response to angiogenic stimuli (35). Growth factors, including VEGF isoforms and basic fibroblast growth factor (bFGF), are important angiogenic stimulators whose receptors have been shown to associate with certain integrins (23, 36, 37). Among these, αvβ3 integrin, in particular, has been shown to interact with VEGFR-2 and PDGFR-β (22). Moreover, the activation of αvβ3 integrin by vitronectin synergistically enhances the VEGF-induced interaction between VEGFR-2 and αvβ3 integrin (38). The interaction between angiogenic growth factor receptors and αvβ3 integrin has been suggested to play an important role in tumor angiogenesis. In particular, molecules that target αvβ3 integrin have been shown to block the interaction between the two receptors, resulting in the inhibition of integrin- and growth factor-induced angiogenesis (23). In the current study, we showed that FAS1-domain protein inhibits VEGF165-induced angiogenesis by directly binding to αvβ3 integrin, inhibiting the association of αvβ3 integrin with VEGFR-2 and attenuating signaling pathways downstream of VEGFR-2, regardless of substrate. These results suggest that FAS1-domain protein targets the interaction between the VEGF receptor and αvβ3 integrin independent of whether αvβ3 integrin is occupied by an extracellular matrix protein such as vitronectin.

In this study, we initially assessed the ability of FAS1-domain protein to inhibit VEGF165-induced angiogenesis, both in vitro and in vivo. Because we had reported previously that FAS1-domain protein inhibits tumor angiogenesis via αvβ3 integrin (11, 39), we hypothesized that the inhibition of VEGF165-induced growth by FAS1-domain protein was dependent on αvβ3 integrin. We tested this hypothesis using HEK293 cells (which lack endogenous αvβ3 integrin and VEGFR-2) co-transfected with VEGFR-2 and β3 or β5 integrin. FAS1-domain protein inhibited VEGF165-induced growth only in HEK293 cells

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expressing both \(\alpha\beta3\) integrin and VEGFR-2 (VEGFR-2/\(\beta3/\)HEK293). Furthermore, we showed that FAS1-domain protein inhibited VEGF\(_{165}\)-induced endothelial cell migration in an \(\alpha\beta3\) integrin-dependent manner. These results indicate that FAS1-domain protein inhibits VEGF-mediated angiogenesis by inhibiting the interaction between VEGFR-2 and \(\alpha\beta3\) integrin.

To gain insight into the molecular mechanisms underlying these observations, we assessed the effect of FAS1-domain protein on the VEGF\(_{165}\)-induced association between VEGFR-2 and \(\alpha\beta3\) integrin. We determined that this association was enhanced after the cells were activated by either VEGF\(_{165}\) or the ligation of \(\alpha\beta3\) integrin to vitronectin. Moreover, the association of VEGFR-2 with \(\alpha\beta3\) integrin was enhanced to a substantial degree in VEGF\(_{165}\)-stimulated cells plated on vitronectin, indicating that \(\alpha\beta3\) integrin participates in the full activation of VEGFR-2. All conditions that enhanced these associations were inhibited by FAS1-domain protein. Furthermore, FAS1-domain protein inhibited VEGFR-2 phosphorylation as well as phosphorylation of the downstream targets, AKT, ERK and p38 MAPK. Inhibition of the association between VEGFR-2 and \(\alpha\beta3\) integrin by FAS1-domain protein appeared to be dependent on the binding of FAS1-domain protein to \(\alpha\beta3\) integrin. Although it may also possibly be due to direct binding of FAS1-domain protein to VEGFR-2, this is unlikely because FAS1-domain protein failed to inhibit the phosphorylation of VEGFR-2 upon VEGF\(_{165}\) stimulation in cells that expressed VEGFR-2 but not \(\alpha\beta3\) integrin. Therefore, we hypothesized that FAS1-domain protein inhibits VEGF\(_{165}\)-induced VEGFR-2 phosphorylation by binding to \(\alpha\beta3\) integrin and interfering with the association between VEGFR-2 and \(\alpha\beta3\) integrin. Using a cell-protein SPR assay with HEK293 cells transfected with VEGFR-2 or \(\beta3\) integrin to test this hypothesis, we determined that FAS1-domain protein bound to \(\beta3/\)HEK293 cells, but not to VEGFR-2/HEK293 cells. These results indicate
that FAS1-domain protein inhibits the association between VEGFR-2 and αβ3 integrin by directly binding to αβ3 integrin. It has been determined that antagonists of αβ3 integrin inhibit angiogenesis in cancer, whereas knockout mice lacking αβ3 integrin not only develop normally, they exhibit enhanced tumor angiogenesis. In fact, in the absence of ligation or when occupied by a soluble ligand, the αβ3 integrin can induce pro-apoptotic signals in endothelial cells. Unligated αβ3 integrin triggers apoptosis in endothelial cells (40). The soluble αβ3 integrin ligand from the alpha 3 chain of type IV collagen, referred to as tumstatin, elicits endothelial cell apoptosis (41). We reported previously that FAS1-domain protein also induces apoptosis not only in endothelial cells but also in αβ3 integrin-expressing tumor cells in an αβ3 integrin-dependent manner (11). Moreover, endothelial cells isolated from β3-null mice exhibit elevated VEGFR-2 expression and activity (42, 43), suggesting that elevated VEGFR-2 levels may facilitate an enhanced response to VEGF in β3-null endothelial cells. Although αβ3 integrin, which is upregulated in newly formed vessels, has long been considered to target angiogenic events, VEGFR-2 could compensate for the blockade of αβ3 integrin. Therefore, simultaneously targeting both αβ3 integrin and VEGFR-2 could be helpful in angiogenesis therapy. From this perspective, FAS1-domain protein, which inhibits not only αβ3 integrin-mediated signaling but also VEGFR-2-mediated signaling, could have potential as a therapeutic agent against tumor angiogenesis. It was also recently reported that activation of αβ3 integrin upregulates VEGF production in tumor cells that express this integrin (44, 45). Although we did not attempt to determine whether FAS1-domain protein blocked VEGF production in tumor cells, it may block αβ3 integrin in tumor cells, resulting in decreased VEGF production. In conclusion, we suggest that FAS1-domain protein is an angiogenesis regulator that targets two closely associated, major angiogenic pathways—the αβ3 integrin- and VEGF-mediated pathways—
highlighting the future potential of FAS1-domain protein as an effective anticancer drug.
Acknowledgement

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References


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Figure legends

FIGURE 1. FAS1-domain protein inhibits VEGF_{165}-induced angiogenesis in vitro and in vivo.

(A-B) Tube formation assay. (A) Serum-starved HUVECs were preincubated with the indicated concentrations of the FAS1-domain protein 30 min prior to stimulation with 50 ng/ml of VEGF_{165}. These cells were then seeded on Matrigel and imaged after 6 to 8 h. (B) Quantitative analysis of the tube formation assay. HPF, high-power field (× 200). Columns, means of triplicate wells. (C-D) Matrigel plug assay. The FAS1-domain protein was mixed with Matrigel coupled with VEGF_{165} (250 ng/ml) and then injected into the flanks of mice. After 9 days, the Matrigel plugs were removed and then stained with anti-CD31 antibody. The number of CD31-positive blood vessels from 4 separate fields (original magnification × 200) were counted and averaged. Results were depicted as means in D. Scale bar represents 100 μm. Each group consisted of five or six Matrigel plugs.; **, P < 0.01; ***, P < 0.001.

FIGURE 2. FAS1-domain protein inhibits VEGF_{165}-induced cell proliferation and migration via the αvβ3 integrin.

(A-B) The averages from three separate determinations using the MTT assay. HUVECs (A), VEGFR-2/β3/HEK293 and VEGFR-2/β5/HEK293 cells (B) were plated on 96-well plates for 12 h, after which the medium was replaced with serum-free medium and the cells were incubated for 24 h. The FAS1-domain protein was added to the cells 30 min prior to stimulation with VEGF_{165} (20 ng/ml) in serum-free media. After 48 h of incubation, cell viability was evaluated using the MTT assay. (C-F) Migration inserts were coated using 2% BSA, 5 μg/ml of collagen type I or vitronectin. Serum-starved HUVECs (C), VEGFR-2/β3/HEK293 and VEGFR-2/β5/HEK293 cells (E) were preincubated with the indicated
concentrations of the FAS1-domain protein for 30 min. These cells were permitted to migrate for 6 h in the presence or absence of 20 ng/ml of VEGF<sub>165</sub>. (D and F) Cell migration was quantified by counting the migrated cells in five high-power fields. The results are expressed as the mean ± SD. from three independent experiments. Statistical analysis of significance by t-test: *, P<0.05; **, P < 0.01; Col., collagen type I; VN, vitronectin.

FIGURE 3. FAS1-domain protein inhibits association of αvβ3 integrin with VEGFR-2 and attenuates VEGF<sub>165</sub>-induced VEGFR-2 phosphorylation.

(A) Serum-starved VEGFR-2/β3/HEK293 cells were plated on collagen-coated dishes in incomplete medium supplemented with the FAS1-domain protein (10 μM) for 30 min and then stimulated for 5 min with VEGF<sub>165</sub> (20 ng/ml) or vehicle alone. The cells were lysed and immunoprecipitated with anti-VEGFR-2 antibody. The immunoprecipitates were analyzed via SDS-PAGE followed by immunoblotting with anti-β3 integrin and anti-VEGFR-2 antibodies. The total cell lysates were immunoblotted with anti-β3 integrin antibody to assess its expression level. Immunoreactive bands were detected by ECL. (B-E) Serum-starved HUVEC, control shRNA/HUVEC, β3 integrin shRNA/HUVEC, VEGFR-2/β3/HEK293, and VEGFR-2/β5/HEK293 cells were plated on collagen-coated dishes in incomplete medium supplement with the FAS1-domain protein (10 μM) for 30 min and then stimulated for 5 min with VEGF<sub>165</sub> (20 ng/ml) or vehicle alone. Cells were lysed and immunoprecipitated with anti-VEGFR-2 Ab. Immunoprecipitates were analyzed via SDS-PAGE followed by immunoblotting with anti-phospho-tyrosine Ab and anti-VEGFR-2 Ab. The total cells were lysed and immunoblotted with anti-β3, anti-β5 integrin, anti-phospho-tyrosine-VEGFR-2 Ab, and anti-β-actin Ab. Immunoreactive bands were detected by ECL. P, Phosphorylated protein; IB, Immunoblotting; IP, Immunoprecipitation.
FIGURE 4. FAS1-domain protein inhibits vitronectin-induced VEGFR-2 phosphorylation and association of αvβ3 integrin with VEGFR-2.

(A-D) Serum-starved HUVEC and VEGFR-2/α3/HEK293 cells were plated on vitronectin-coated dishes in incomplete medium supplemented with the FAS1-domain protein (10 μM) for 30 min, then stimulated for 5 min with VEGF165 (20 ng/ml) or vehicle alone. The cells were lysed and immunoprecipitated with anti-VEGFR-2 antibody. Immunoprecipitates with anti-VEGFR-2 antibody were analyzed via SDS-PAGE followed by immunoblotting with anti-phospho-tyrosine and anti-VEGFR-2 antibodies (A and B) or with anti-β3 integrin and anti-VEGFR-2 antibodies (C and D). Immunoreactive bands were detected by ECL.

FIGURE 5. FAS1-domain protein interacts with αvβ3 integrin but not with VEGFR-2.

(A) Diagram of cell-protein SPR analysis. Suspended cells (400,000 cells/ml) were injected at a flow rate of 25 μl/min for 10 min. HEK293 cells, which were used as a control were passed over the right channel and β3/HEK293 or VEGFR-2/HEK293 cells, were passed over the left channel, respectively. Then PBS (None) or 7.8 μM of FAS1-domain protein were injected at a flow rate of 25 μl/min for 7 min. (B) The expression of β3 integrin and VEGFR-2 was assessed by Western blot analysis. HEK293, β3/HEK293, and VEGFR-2/HEK293 cells were lysed and immunoblotted with anti-β3 and anti-VEGFR-2 antibodies. β-actin was used as a loading control. (C-D) Sensorgram shows the binding of FAS1 domain to β3/HEK293 (C) and VEGFR-2/HEK293 cells (D). The activated mSAM 10% COOH surface was coated with a 50 μg/ml of poly-L-Lysine in 10 mM sodium acetate buffer, pH 4.0, and was blocked with 1M ethanolamine hydrochloride at a flow rate of 25 μl/min. Suspended cells were injected for 10 min at a flow rate of 25 μl/min. HEK293 cells, which were used as a control were passed over the right channels and β3/HEK293 or VEGFR-2/HEK293 cells, were passed over the
right channel, respectively. Then PBS (None) or 7.8 μM of FAS1-domain protein were injected for 7 min to monitor the interaction. All sensorgrams were obtained by subtracting the binding of the FAS1-domain protein to the HEK293 control cells. Specific binding of the FAS1-domain protein to each cell was calculated from the difference between FAS1-domain protein and PBS binding. $K_d$, equilibrium dissociation constant; n.d., not detected.

FIGURE 6. FAS1-domain protein inhibits the VEGF_{165}-induced signaling pathways.

Serum-starved VEGFR-2/β3/HEK293 cells were plated on vitronectin or collagen type I-coated dishes in incomplete medium supplemented with the FAS1-domain protein (10 μM) for 30 min and then stimulated for 5 min with VEGF_{165} (20 ng/ml) or vehicle alone. These cells were lysed and examined by the Western blot analysis using antibodies against the indicated protein (P, antibody specific for phosphorylated protein).
Legends to Supplemental Figures


The HUVECs were suspended in M199 medium containing 0.5% FBS and treated for 1h and 6h at 37 °C with the indicated FAS1-domain protein concentrations. The percentage of live cells was determined using a Luna™ automated cell counter (Logos Biosystems, Inc.) with 0.4% trypan blue stain. The results were expressed as the mean ± SD. from three independent experiments.

SUPPLEMENTAL FIGURE 2. FAS1-domain protein inhibits VEGF165-induced association of αβ3 integrin with VEGFR-2 and VEGFR-2 phosphorylation via αβ3 integrin. (A) Band intensities of β3 integrin immunoprecipitated with anti-VEGFR-2 antibody were obtained by densitometry and then normalized to total anti-VEGFR-2 signals and relative intensities were expressed in arbitrary units in which the intensities of VEGF-treated band levels was set to 100%. (B) Co-immunoprecipitation assay with normal rabbit-IgG and anti-VEGFR-2 antibodies in VEGFR-2/β3/HEK293 cells. Serum-starved VEGFR-2/β3/HEK293 cells were plated on collagen type I-coated dishes in serum-free medium, followed by stimulation for 5 min with VEGF165 (20 ng/ml) or vehicle alone. The cells were then lysed and immunoprecipitated with anti-VEGFR-2 antibody or normal rabbit-IgG. Immunoprecipitates were analyzed via SDS-PAGE followed by immunoblotting with anti-VEGFR-2 and anti-β3 antibodies. IB, immunoblotting; IP, immunoprecipitation. (C-E) Relative band intensities of phospho-VEGFR-2 obtained by densitometry were normalized to the total VEGFR-2 band, and relative intensities were expressed in arbitrary units in which the intensity of VEGF-treated band level was set to 100.

SUPPLEMENTAL FIGURE 3. FAS1-domain protein inhibits both vitronectin- and
collagen-induced VEGFR-2 phosphorylation and association of αvβ3 integrin with VEGFR-2.

(A-B). Relative band intensities of phospho-VEGFR-2 obtained by densitometry were normalized to total VEGFR-2 band, and relative intensities were expressed in arbitrary units in which the intensity of VEGF-treated band level was set to 100. (C) Serum-starved VEGFR-2/β3/HEK293 cells were plated on collagen-coated dishes, and then stimulated for 5 min with VEGF165 (20 ng/ml) or vehicle alone. The cells were lysed and immunoprecipitated with anti-VEGFR-2 Ab. Immunoprecipitates with anti-VEGFR-2 Ab were analyzed via SDS-PAGE followed by immunoblotting with anti-phospho-tyrosine and anti-VEGFR-2 Ab. Immunoreactive bands were detected by ECL. (D-E). Band intensities of β3 integrin immunoprecipitated with anti-VEGFR-2 antibody were obtained by densitometry and then normalized to total anti-VEGFR-2 signals and relative intensities were expressed in arbitrary units in which the intensities of VEGF-treated band levels was set to 100%.

SUPPLEMENTAL FIGURE 4.

Serum-starved VEGFR-2/β5/HEK293 cells were plated on collagen I-coated in serum-free medium supplemented with FAS1-domain protein (10 μM) for 30 min, followed by stimulation for 5 min with VEGF165 (20 ng/ml) or vehicle alone. These cells were then lysed and analyzed via Western blotting using antibodies against the indicated proteins (p, antibody specific for phosphorylated protein).
Nam et al., Fig. 1A-B
None

VEGF

VEGF + FAS1 (5 μM)

VEGF + FAS1 (10 μM)

The number of blood vessels/HPF

VEGF (250 ng/ml)

FAS1 (μM)

- - 5 10

0 10 20 30 40 50 60

** ***

Nam et al., Fig. 1C-D
Nam et al., Fig. 2A-B

A

B

HUVEC

VEGF (20 ng/ml)

FAS1 (μM)

Absorbance at 570nm

0.30

0.25

0.20

0.15

0.10

0.05

0.00

- - 2.5 5 10 20

VEGF (20 ng/ml)

FAS1 (μM)

Absorbance at 570nm

0.8

0.6

0.4

0.2

0.0

- - 2.5 5 10 20

VEGF (20 ng/ml)

FAS1 (μM)

VEGFR-2/β3/HEK293

VEGFR-2/β5/HEK293

* * * * * *
C

Migration Assay (The number of cells/HPF)

D

Coating: BSA  Col.  VN

FAS1 (20 μM)  VEGF (20 ng/ml)

Nam et al., Fig. 2C-D
$\text{VEGFR-2}/\beta_3/\text{HEK}$ vs $\text{VEGFR-2}/\beta_5/\text{HEK}$

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Coating: Col.

Figure 2E-F

Nam et al., Fig. 2E-F
**D**

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IP: VEGFR-2 Ab

P-Tyr IB

VEGFR-2 IB

β3 IB

Total lysate

**E**

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IP: VEGFR-2 Ab

P-Tyr IB

VEGFR-2 IB

β5 IB

Total lysate
Nam et al., Fig. 4A-D
A Cell-Protein SPR analysis

- **Analyte** (25 μl/min)
  - FAS1-domain

- **Immobilization**
  - Left channel
    - αvβ3/HEK293
    - VEGFR-2/HEK293
  - Right channel
    - HEK293

B HEK293 cells

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C 

\[ K_d = 0.96 \pm 0.14 \mu M \]

Response (μRU) vs. Time (Seconds)

[αvβ3/HEK293 cells]

D 

\[ K_d = n.d. \]

Response (μRU) vs. Time (Seconds)

[VEGFR-2/HEK293 cells]

Nam et al., Fig. 5A-D
Nam et al., Fig. 6
FAS1-domain protein inhibits VEGF165-induced angiogenesis by targeting the interaction between VEGFR-2 and β3 integrin

Ju-Ock Nam, Hye-Nam Son, Eunsung Jun, et al.

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