

Anti-angiogenesis mediated by angiostatin K1–3, K1–4 and K1–4.5

Involvement of p53, FasL, AKT and mRNA deregulation

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Summary

The molecular mechanism mediated by multiple forms of angiostatin via acting on proliferating vascular endothelium remains elusive. To address whether three forms of angiostatin, K1–3, K1–4 or K1–4.5, utilized similar or distinct pathways to mediate anti-angiogenesis, we adopted an adenoviral expression system to express secretable angiostatin molecules for CM collection. The anti-angiogenic activity of K1–3, K1–4 or K1–4.5 was confirmed by using proliferation, migration, tube formation and apoptotic assays of human endothelial cells. These angiostatin molecules at comparable expression level inhibited various *in vitro* angiogenesis assays with some variations. Furthermore, K1–3, K1–4 or K1–4.5 increased the expression of p53 protein

and its downstream effectors, enhanced FasL-mediated signaling pathways, and decreased activation of AKT. At least three different receptors, Fas, integrin $\alpha v \beta 3$ and ATP synthase, were involved in the anti-angiogenic action of angiostatin molecules. Besides, the expression of 189 genes at mRNA level was significantly altered by K1–3, K1–4 or K1–4.5. More than 70% of these genes participate in growth, inflammation, apoptosis, migration and extracellular matrix. Taken together, K1–3, K1–4 and K1–4.5, regardless of the number of kringles in the angiostatin molecules, mediated anti-angiogenesis via mostly similar pathways. We are the first to demonstrate the involvement of DAPK1 in the mediation of anti-angiogenesis by angiostatin.

Keywords

Angiostatin, endothelial cells, AKT, p53, FasL

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Introduction

Tumor growth and metastasis depend on the angiogenic process. Angiogenesis, formation of new blood vessels from pre-existing vasculature, is a balanced process tightly regulated by angiogenic inducers and inhibitors (1). Angiogenic inhibitors directly or indirectly targeting at endothelial cells thus provide a complementary approach in conjunction with traditional cancer therapy for the treatment of various types of human cancer (2, 3). Angiostatin is one of the few circulating angiogenic inhibitors identified in the serum and urine of tumor-bearing animals.

Angiostatin, consisting of the first four kringle domains of plasminogen, K1–4, suppresses angiogenesis and tumor growth in mice (4). Another form of angiostatin molecule consisting of only the first three kringle domains (K1–3) was later shown to manifest a stronger or compatible inhibitory effect on angiogenesis *in vitro* and *in vivo* (5, 6). K1–3 is the form of angiostatin

molecule tested in clinical trials for cancer treatment. Later on, another form of angiostatin molecule consisting of K1–4 and 85% of K5 (K1–4.5) was found to be a naturally occurring intermediate via autoproteolysis of plasminogen (7). Moreover, plasmin-activated angiostatin K1–5, at 50-fold lower dose than K1–4, was shown to suppress angiogenesis and tumor growth (8). Although multiple forms of angiostatin have been identified to date, the efficacy of anti-angiogenesis exerted by angiostatin is still under intensive investigation in both preclinical and clinical settings.

The inhibitory effect exerted by multiple forms of angiostatin is mostly restricted to endothelial cells. K1–3 inhibits endothelial cell proliferation by disrupting the G2/M transition in the cell cycle progression (9). K1–4 induces bovine capillary endothelial cells to undergo apoptosis via activation of focal adhesion kinase (10). K1–4 diminished the MAPK activation induced by angiogenic inducers or induced the expression of E-

selectin (11, 12). Inhibition of endothelial cells by K1-4 is also accompanied by down-regulation of cell cycle regulatory protein Cdk5 (13). In addition, K1-3 is a novel anti-inflammatory factor by inhibiting leukocyte recruitment (14). Although angiostatin has previously shown to be an endothelial cell-specific inhibitor for angiogenesis, it was recently shown that angiostatin inhibits hepatocyte growth factor-mediated proliferation and signaling in both vascular endothelial and smooth muscle cells while having no effect on bFGF- or VEGF-induced HUVEC proliferation (15). Multiple inhibitory effects of various angiostatin molecules suggest that multiple cellular components or signaling pathways are involved in mediation of the inhibitory effect exerted by these molecules.

At least two types of cell surface receptors are bound by angiostatin molecules. First, K1-3 blocks the activity of ATP synthase via direct binding to this complex residing on endothelial cell surface (16, 17). Another potential receptor for K1-3, K1-4 and K1-5 was integrin $\alpha v \beta 3$ using bovine endothelial cells as a target cell (18). Third, membrane-associated actin was required for generation of K1-4.5 (19). Taken together, multiple receptors may be required for multiple forms of angiostatin to mediate anti-angiogenesis.

The anti-angiogenic effect of angiostatin molecules is primarily through induction of apoptosis (20). The apoptotic pathways triggered by these molecules include activation of several caspases, stimulation of FasL-mediated extrinsic signaling molecules, up-regulation of p53 protein and mitochondria dysfunction followed by the release of cytochrome C (21-23). However, none of these studies was able to compare the similarities or differences of different forms of angiostatin in the same studies. Whether these molecules trigger similar or distinct pathways to mediate anti-angiogenic effects remains to be clarified.

Since the molecular mechanism of angiostatin that acts on endothelium remains elusive, we used adenovirus to overexpress three secretable forms of angiostatin molecules, K1-3, K1-4 and K1-4.5. Angiostatin containing conditioned media or direct infection of HUVECs with angiostatin-expressing adenovirus were used to study the effect of angiostatin on endothelial cells. The involvement of three apoptotic signaling pathways was examined by Western blot analysis. Neutralization of ATP synthase, integrin $\alpha v \beta 3$ or Fas by antibodies was investigated for their roles in transducing the anti-angiogenic signaling mediated by these three angiostatin molecules. Meanwhile, the gene expression profiles of VEGF-treated proliferating HUVECs under the influence of K1-3, K1-4 or K1-4.5 were compared using cDNA microarray analysis. Distinct and common pathways shared by these three molecules were discussed.

Materials and methods

Materials

Adenovirus expression vector and Gigapack III XL-4 kits were from TaKaRa (Shiga, Japan) and Stratagene (La Jolla, CA, USA), respectively. Human recombinant VEGF-A, consisting of 165 amino acids and an anti-human caspase 8 antibody recognizing precursor and 42 kDa doublets, were from R&D System (Minneapolis, MN, USA). Endothelial growth medium 2

(EGM2) was from BioWittaker (Walkersville, MD, USA). FBS, BSA, gelatin, and all other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA). Lysine-sepharose was purchased from Amersham Biosciences (Uppsala, Sweden). Cell titer 96[®] One Aqueous One Solution Cell Proliferation Assay (MTS) kit, reagents and enzymes for molecular biology were from Promega (Madison, WI, USA). Polyvinylpyrrolidone-free polycarbonate membrane for migration assays was ordered from Neuro Probe Inc. (Gaithersburg, MD, USA). Matrigel was from BD Bioscience (Bedford, MA, USA). Annexin V-FITC and PI apoptosis kit was from Stroagbiotech (Taipei, Taiwan). Oligonucleotide primers for PCR were from MDBio (Taipei, Taiwan). Antibodies to phospho-AKT (Ser473) and AKT were from Cell Signaling technology (Beverly, MA, USA). Antibodies to FasL and Bax were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to p53 and p21 were from Oncogene (Boston, MA, USA). An antibody to α -tubulin was from Neo Markers (Fremont, CA, USA). Neutralization antibodies to integrin $\alpha v \beta 3$ (LM609) and Fas (clone ZB4) were from Chemicon (Temecula, CA, USA) and Upstate Biotechnology (Lake Placid, NY, USA), respectively. A monoclonal anti- α -ATP synthase antibody was from Molecular Probes (Eugene, OR, USA). An antibody to K1-3 was kindly provided by Dr. Ming T. Lin at Tzu Chi University (Taiwan). Renaissance Chemiluminescence Reagent Plus was from NEN Life Science Products (Boston, MA, USA). North2South Chemiluminescent Hybridization and Detection kits for Southern blotting were from Pierce (Rockford, IL, USA). TRIzol reagent was from Invitrogen (Carlsbad, CA, USA). Biotin-dUTP was from Roche (Germany). Geimsa staining solution was from Merck (Germany).

Cell culture

HUVECs were isolated as previously described (24). Following isolation, pooled HUVECs from three donors were seeded in gelatinized dishes containing EGM-2 and were used at early passages. Both human lung carcinoma A549 and promyelocytic leukemia HL-60 cells were cultured as described by American Tissue Culture Collection. Human kidney embryonic 293 cells at no later than 30 passages were maintained in MEM supplemented with 10% heat-inactivated horse serum, 1 mM sodium pyruvate, 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Establishment and titration of recombinant adenovirus

Replication-deficient recombinant adenoviruses expressing K1-3, K1-4, or K1-4.5 were generated using Adenovirus Expression Vector kit as described by the manufacturer (TaKaRa, Japan). Briefly, cDNA fragments encoding K1-3 (amino acids 1-352), K1-4 (amino acids 1-454) and K1-4.5 (amino acids 1-549) were PCR-amplified with specific primers for each fragment and cloned into a cosmid vector. The primer design was based on the accession number NM_000301 for human plasminogen mRNA. Adenoviruses expressing K1-3 (AdK1-3), K1-4 (AdK1-4), K1-4.5 (AdK1-4.5) and empty virus (Adnull) were obtained by homologous recombination between the cosmid vector bearing K1-3, K1-4, or K1-4.5 cDNA and genomic DNA-terminal protein complex in human embryonic kidney 293 cells. Following viral amplification in 293 cells, virus titers were determined by calculating the 50% infectious dose.

Collection of CM following adenoviral infection of A549 cells

Adenoviral infection of A549 cells at the indicated MOI was carried out in serum-free medium for 1 h at 37°C. Following infection, cells were washed and incubated for 4 days with serum-free medium. CM derived from 4-day infection of Adnull, AdK1-3, AdK1-4 or AdK1-4.5 were prepared by filter-sterilization and subsequent UV-irradiation at 2400 mJ to inactivate any contamination of live viral particles (25).

Immunoblotting of K1-3, K1-4, and K1-4.5 in the CM

Two hundred microliters of CM were precipitated overnight at 4°C with 50 µl lysine-sepharose. The precipitated protein complex was fractionated on a 10% SDS-PAGE under non-reducing conditions followed by overnight transferring to PVDF membrane. The membrane was first probed with a rabbit polyclonal antibody to K1-3 (1:20,000) and then with horseradish peroxidase-conjugated anti-rabbit antibody followed by detection with Chemiluminescence Reagent Plus.

Cell proliferation assay

HUVECs were seeded at a density of 5,000 cells/well in a gelatin-coated 96-well tissue culture plate. Twelve hours following seeding, HUVECs were serum-deprived for 12 h in the starvation medium consisting of M199, 1% heat-inactivated FBS, and 0.1% BSA. Serum-deprived cells were treated with M199-diluted CM containing final concentrations of 5% heat-inactivated FBS, 5 ng/ml VEGF-A and 0.1% BSA. For receptor neutralization, serum-starved cells were pre-incubated for 1 h with 2.5 µg/ml anti-integrin $\alpha\text{v}\beta\text{3}$ antibody, 1 µg/ml anti-Fas antibody, or 2.5 µg/ml anti- α -ATP synthase antibody prior to the treatment. Cell proliferation was measured using MTS kits at 48 h post-treatment. Each treatment was tested in quadruplicate. The experiments were repeated twice.

Cell migration

Cell migration was measured in a Boyden chamber by using 8 µm polyvinylpyrrolidone-free polycarbonate membrane coated with 0.1% gelatin. The membrane was placed over bottom chambers filled with treatment medium containing 75% CM and final concentrations of 5% heat-inactivated FBS, 5 ng/ml VEGF-A and 0.1% BSA. HUVECs at a density of 2,500 cells in M199 medium were added to upper chambers. After incubation at 37°C for 6 h, the membrane was stained with Geimsa then analyzed for the number of stained cells that had migrated to the opposite side of the membrane. The experiment was repeated twice.

Tube formation assay

HUVECs (4.6×10^4 per well) were seeded in duplicates onto 48-well culture dishes coated with Matrigel (13.4 mg/ml) followed by treatment medium containing 75% CM and final concentrations of 5% heat-inactivated FBS, 5 ng/ml VEGF-A and 0.1% BSA for 16 h. Tube formation was observed by an inverted Olympus phase-contrast microscope and five high power fields at 100 X magnification were randomly taken by using Olympus DP12 digital camera. The number of tubes for each treatment was quantified by software developed by Dr. Y. N. Sun at National Cheng Kung University. This experiment was independently repeated three times.

Apoptosis assay by flow cytometry

Subconfluent HUVECs were infected with indicated adenovirus at MOI of 100. Following 1-h infection, cells were treated for 48 hours with M199-based growth medium containing and final concentrations of 5% heat-inactivated FBS, 5 ng/ml VEGF-A and 0.1% BSA. Serum-deprived HUVECs for 16 h were used as a positive control, whereas EGM-2 treated cells served as a negative control. For receptor neutralization, serum-starved cells were pre-incubated for 1 h with 2.5 µg/ml anti-integrin $\alpha\text{v}\beta\text{3}$ antibody, 1 µg/ml anti-Fas antibody, or 2.5 µg/ml anti- α -ATP synthase antibody prior to 48-h incubation of treatment medium containing 75% CM and final concentrations of 5% heat-inactivated FBS, 5 ng/ml VEGF-A and 0.1% BSA. Treated cells were washed and labeled with annexin V-FITC and PI apoptosis kits. The cells were then analyzed by FACSCalibur flow cytometer (BD Biosciences). Each treatment experiment was independently repeated two to three times.

Western blot analysis

Serum-deprived subconfluent HUVECs were treated for 24 h with treatment medium containing 75% CM and final concentrations of 5% heat-inactivated FBS, 5 ng/ml VEGF-A and 0.1% BSA. Cells were homogenized in the appropriate lysis buffer containing protease inhibitors. The protein concentration in each lysate was measured by Bradford protein assay. Equal amounts of total protein were separated by SDS-PAGE and then blotted onto a PVDF membrane. Protein blots were hybridized with the indicated primary antibody and then with secondary antibody, followed by detection with Renaissance Chemiluminescence Reagent Plus.

RT-PCR and Southern blot

Following treatment and isolation of DNA-free total RNA, 1 µg of total RNA was used as a template for reverse transcription using oligo(dT) primers. The cDNA mixture was then used as a template for gene-specific PCR. GAPDH was an internal control. Gene-specific primers and their PCR product for DAPK1, c-FLIP, SELE, AXL, TIE1, PIM1, SMAD4, PPP2R2A, and GAPDH are listed in supplementary Table 1 (available online at www.thrombosis-online.com). The amplification cycle for each gene fragment was in the linear range. In the case of c-FLIP mRNA semi-quantification, co-amplified PCR products were fractionated by electrophoresis then blotted onto nitrocellulose membrane. The PCR product specific to c-FLIP on the membrane was detected using North2South Chemiluminescence Nucleic Acid Hybridization and Detection kit followed by autoradiography.

cDNA microarray analysis

Total RNA was isolated using TRIzol reagent from HUVECs treated for 4 h with treatment medium containing 75% CM of null, K1-3, K1-4 or K1-4.5 and final concentrations of 5% heat-inactivated FBS, 5 ng/ml VEGF-A and 0.1% BSA. Total RNA (12.5 µg) was labeled with biotin-dUTP during reverse transcription for cDNA hybridization analysis as previously described (24). The cDNA chips of 6,388 human unigenes were used for hybridization. A detailed gene list of this chip is listed at http://web.nycu.edu.tw/~chingli/personal/biochip/chinese/cht_

total.htm. Following hybridization and signal detection by Powerlook 3000 dpi scanner, the signal intensity of each gene was analyzed using Gene Spring 7.0 software (Silicon Genetics, USA). Each treatment condition was independently repeated three times.

HL-60 cell adhesion assay

Subconfluent HUVECs were plated on gelatin-coated 3.5-cm dishes until reaching confluence. Monolayers were treated for 16 h with treatment medium containing 75% CM of null, K1-3, K1-4 or K1-4.5 and final concentrations of 5% heat-inactivated FBS, 5 ng/ml VEGF-A and 0.1% BSA prior to adhesion assay. HUVECs treated for 4 h with 100 ng/ml LPS prior to adhesion assay served as a positive control. The treatment media was then removed and washed once with RPMI medium with or without 2.5 mM EGTA. Washed HL-60 cells were resuspended in RPMI medium with or without 2.5 mM EGTA. EGTA-treated or untreated HL-60 at 2×10^6 cells in 0.6 ml were added onto the CM- or LPS-primed HUVEC monolayers. Following incubation at 4°C on a rocking platform for 45 min and extensive washes, attached HL60 cells on a HUVEC monolayer were fixed with 2.5% glutaraldehyde. Cells were photographed at 100 X magnification using an Olympus digital camera on an Olympus microscope. Bound HL-60 cells were counted in 10 randomly selected fields. The number of bound cells was expressed as a mean number of bound cells/cm² ± standard deviation (SD). This experiment was independently repeated two times.

Statistical analysis

Data were compared using the Student's t test and one way ANOVA as appropriate. P values of < 0.05 were regarded as significant.

Results

Protein levels and functions of K1-3, K1-4 and K1-4.5 in CM

The anti-angiogenic ability of K1-3, K1-4, and K1-4.5 was never compared in the same study. To interrogate the function and potency of different angiostatin molecules in secretable forms, adenoviral expression vectors encoding a signal peptide of plasminogen and each of angiostatin molecules, K1-3, K1-4 and K1-4.5, were made and termed as AdK1-3, AdK1-4 and AdK1-4.5. Empty virus (Adnull) was used as a negative control. A MOI of 10–100 (30–100% infection efficiency) was used to infect A549 for collection of CM. K1-3, K1-4 or K1-4.5 in the CM was precipitated with lysine sepharose followed by Western blot analysis. The amounts of K1-3, K1-4 and K1-4.5 at predicted molecular weight were not only comparable but also dose-dependently increased with the increments of MOI (Fig. 1A). No specific band was detected in the CM of Adnull infection. In comparison with a known concentration of purified human angiostatin, the amount of K1-3, K1-4 and K1-4.5 proteins in the CM was around 20 µg/ml, which fell or exceeded the range for the reported biological activity of these molecules (6, 22). CM collected from a MOI of 100 was used for the following studies.

Angiostatin not only inhibits VEGF-induced HUVEC proliferation and migration but also induces apoptosis (5, 6, 20). Ef-

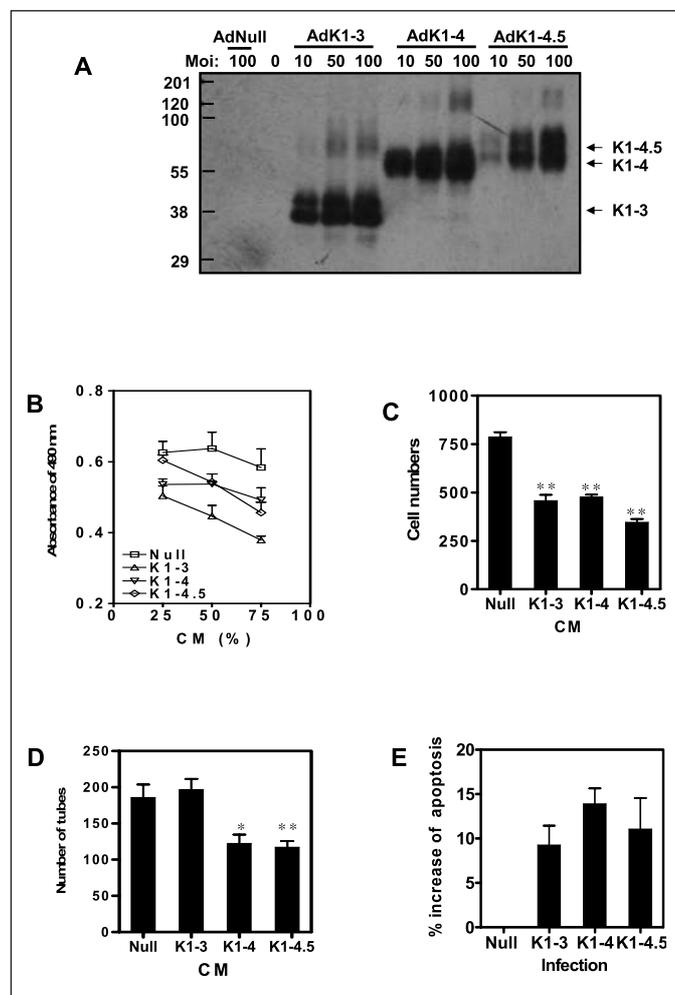


Figure 1: K1-3, K1-4 or K1-4.5 inhibits proliferation, migration, and tube formation and induce apoptosis. A) CM was collected from A549 cells at 96 h after infection of Adnull, AdK1-3, AdK1-4, or AdK1-4.5 at MOI of 10, 50 and 100 followed by lysine-sepharose precipitation and Western blot analysis. Arrows indicate K1-3, K1-4 and K1-4.5. Expression level of these molecules increases with the increment of MOI. B) Serum-starved HUVECs were treated for 48 h with treatment medium of null control, K1-3, K1-4 and K1-4.5. Following treatment, cell proliferation was measured by MTS assay and expressed as absorbance of 490 nm ($p < 0.05$ vs. null control). C) Blue stained cells on the opposite side of filter following treatment-induced migration for 6 h was enumerated. Five high power fields were chosen to represent the migratory cells for each treatment. ** $p < 0.01$ vs. null control. D) HUVECs seeded on Matrigel were incubated for 16 h with K1-3, K1-4 or K1-4.5. The number of tubes formed in each treatment was the average of three independent experiments and expressed as mean \pm S.D. * $p < 0.05$ and ** $p < 0.01$ vs. null control. E) Subconfluent HUVECs infected with indicated viruses at MOI of 100 were treated for 48 h with M199-based growth medium containing 5% heat-inactivated FBS, 5 ng/ml VEGF-A and 0.1% BSA. Following treatment, cells were harvested for PI and annexin V staining. The data are shown as the average of three independent experiments (mean \pm S.D.).

fects of K1-3, K1-4 and K1-4.5 on the ability of HUVECs to proliferate, migrate, and form tubes were compared. HUVEC proliferation was significantly attenuated in a dose-dependent manner by K1-3, K1-4, and K1-4.5 compared to null control

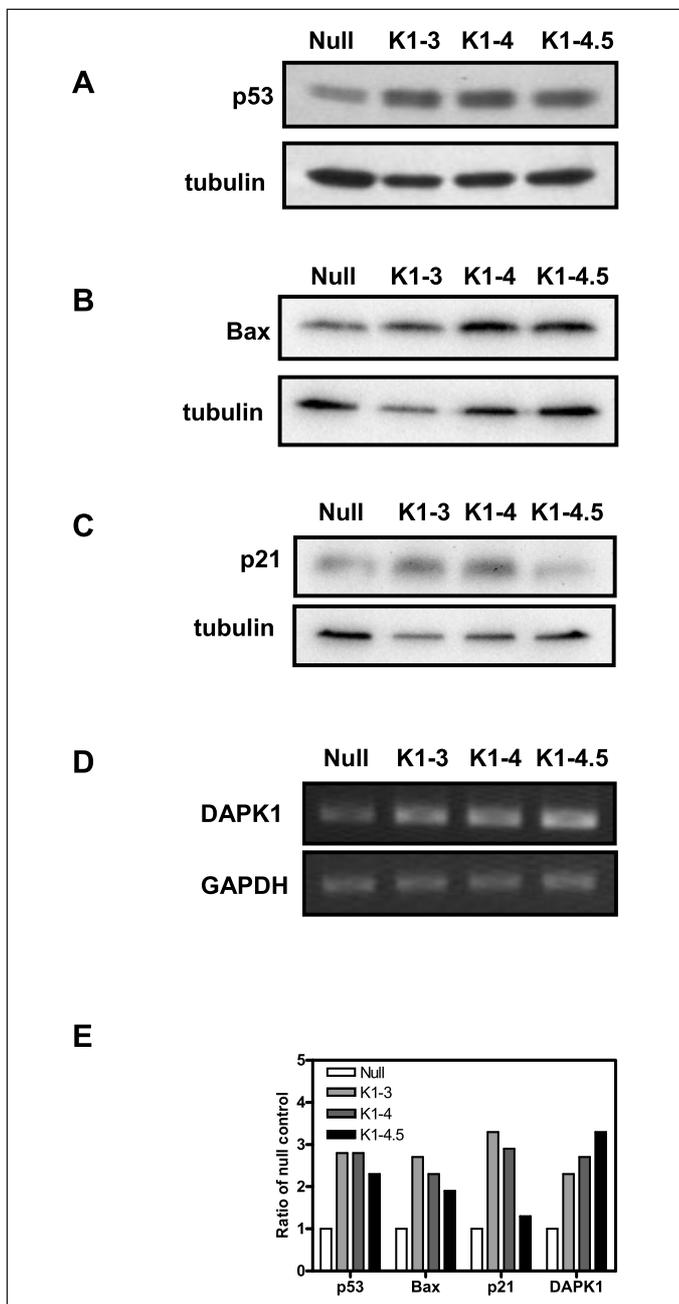


Figure 2: K1-3, K1-4 or K1-4.5 enhances the expression of p53, p21, Bax and DAPK1. Serum-starved HUVECs were exposed for 24 h to indicated form of angiostatin. Western blot analysis was used to detect expression levels of p53 (A), Bax (B), p21 (C). α -tubulin serves as a loading control. D) RT-PCR was used to detect DAPK1 mRNA. GAPDH serves as a loading control. E) A quantitative result of Western blotting and RT-PCR analyses is expressed as ratio of null control.

(Fig. 1B, $P < 0.05$ vs. null control). Since 75% of CM had the highest inhibitory effect on cell proliferation, we then used this percentage of CM to perform migration and tumor formation assays. K1-3, K1-4 and K1-4.5 strongly inhibited the ability of HUVEC to migrate (Fig. 1C). Tube formation assay indicated that K1-4 and K1-4.5 manifested a negative effect on HUVECs to form tubes in Matrigel (Fig. 1D). Surprisingly, no inhibitory effect of K1-3 on tube formation could be detected.

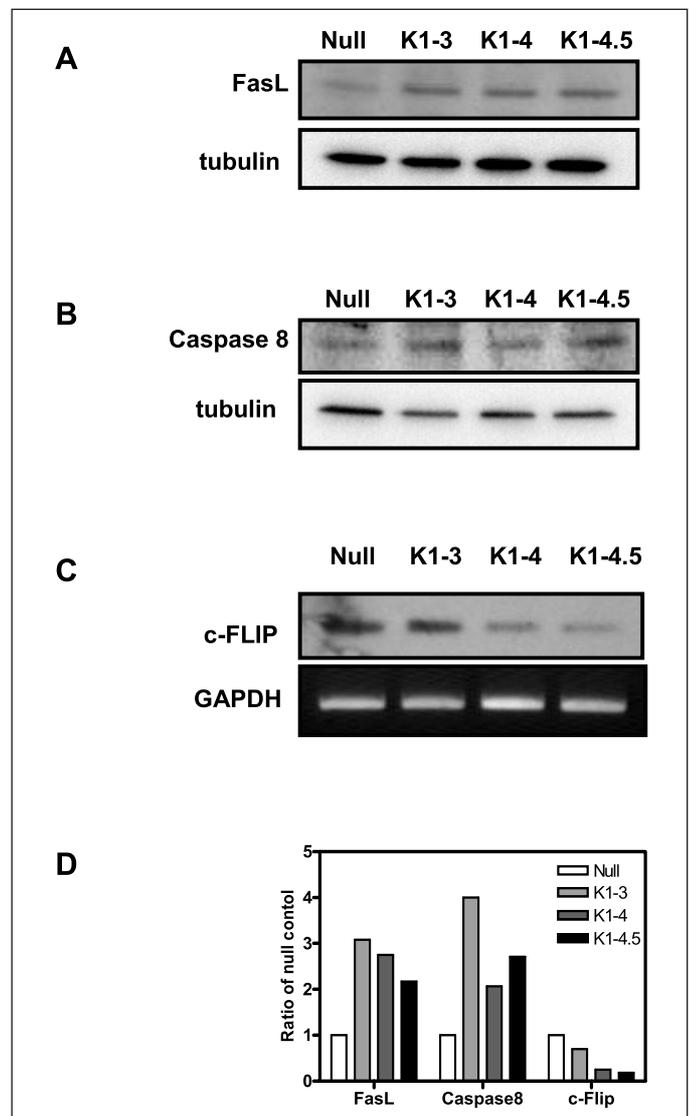


Figure 3: K1-3, K1-4 or K1-4.5 increases the level of FasL protein and cleavage of caspase 8 while reducing cFLIP mRNA expression. Serum-starved HUVECs were exposed for 24 h to the indicated form of angiostatin. Protein lysates were subjected to Western blot analysis using specific antibodies to FasL (A) and cleaved caspase 8 (B). α -tubulin serves as a loading control. C) RT-PCR and Southern blot analysis were used to detect c-FLIP mRNA level. GAPDH serves as a loading control. D) A quantitative result of mRNA expression ratio of c-FLIP vs. GAPDH is expressed as a ratio of null control.

The ability of these molecules to induce apoptosis was examined using flow cytometry. Following direct infection of HUVECs with recombinant adenoviruses at MOI of 100, we routinely observed a 13–18% increase of apoptotic HUVECs induced by K1-3, K1-4, and K1-4.5 compared with null control (Fig. 1E). Angiostatin molecules produced in CM are functional with similar inhibitory effects in most assays except tube formation assay.

K1-3, K1-4 or K1-4.5 induce the expression of p53, Bax, p21, and DAPK1

P53 is a key protein involved in cell cycle control and apoptosis and activates the expression of mitochondrial protein Bax and

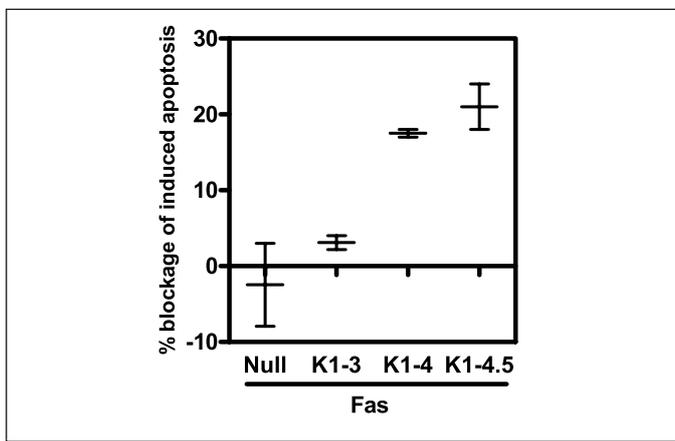


Figure 4: Neutralization of Fas attenuates the pro-apoptotic ability of angiostatin molecules to different extents. Serum-starved HUVECs were pretreated for 1 h with Fas neutralizing antibodies prior to 48-h incubation with treatment medium containing K1-3, K1-4 and K1-4.5. Following treatment, both floating and adherent cells were harvested for apoptotic assay using flow cytometry. Similar data were obtained from three independent experiments and expressed as % blockage of apoptosis. Blockage of apoptosis represents the percent decrease of angiostatin-induced apoptotic cells protected by Fas neutralizing antibody compared to total apoptotic cells induced by angiostatin in the absence of neutralizing antibodies. An average, including the range, of two independent experiments for each treatment is shown.

DAPK1 (26, 27). To examine whether p53 and its downstream targets were involved in the inhibitory effects mediated by K1-3, K1-4 and K1-4.5, cellular proteins or total RNA were harvested from angiostatin-treated proliferating HUVEC for Western blot or RT-PCR analyses. All three forms of angiostatin significantly enhanced the level of p53 protein compared to null control (Fig. 2A). Consistent with induction of p53, the expression of p53 downstream targets, Bax, p21 and DAPK1, was also increased in angiostatin-treated cells (Fig. 2B-D). Together, up-regulation of p53 and its downstream targets are involved in anti-angiogenic action of K1-3, K1-4 and K1-4.5.

Participation of FasL, c-FLIP, and caspase 8 in anti-angiogenesis mediated by K1-3, K1-4 and K1-4.5

Apoptosis is the primary mechanism of anti-angiogenic effect mediated by angiostatin and its related molecules. Multiple death pathways involved in caspases and FasL/cFLIP have been reported in endothelial apoptosis (21, 23, 28). None of these studies directly addressed the difference and similarity of signaling pathways mediated by three different forms of angiostatin. To determine whether K1-3, K1-4 and K1-4.5 mediated similar or distinct apoptotic signaling pathways, Western blot analysis of cell lysates or RT-PCR and Southern blot analysis of total RNA prepared from HUVEC treated for 24 h with null control, K1-3, K1-4 or K1-4.5 in the presence of VEGF were performed. Increased FasL protein and enhanced production of active caspase 8 (42 kDa) were observed in HUVECs treated with K1-3, K1-4, or K1-4.5 relative to null control (Fig. 3A, B). By contrast, the mRNA of c-FLIP was attenuated by these three molecules (Fig. 3C). The extent of c-FLIP mRNA decreased by K1-3 was the least among the three molecules. Together, K1-3, K1-4 and

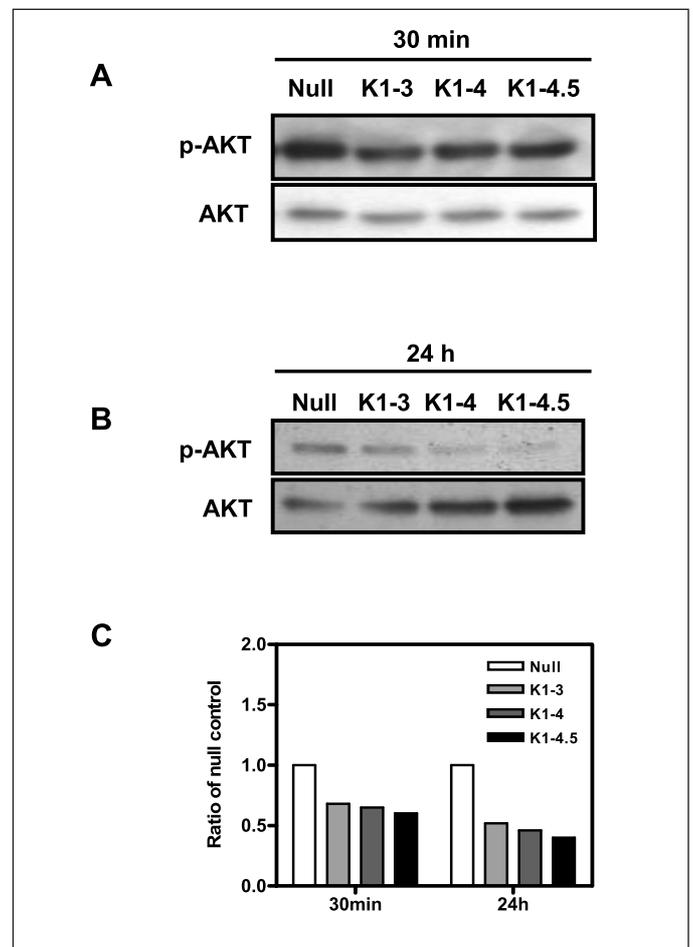


Figure 5: K1-3, K1-4 or K1-4.5 attenuates the activity of AKT. Serum-starved HUVECs were treated for 30 min (A) or 24 h (B), respectively, with K1-3, K1-4 or K1-4.5 followed by cell lysate isolation. Western blot analysis of cell lysates using antibodies specific to serine⁴⁷³ phosphorylation of AKT and total AKT were used to detect the AKT activation status. α -tubulin on the bottom panels serves as a loading control. C) A quantitative result of phosphorylated AKT vs. AKT is expressed as a ratio of null control.

K1-4.5 mediated anti-angiogenesis via increased expression of FasL, cleavage of procaspase 8 into caspase 8 and reduced c-FLIP mRNA.

Neutralization of FasL attenuated the ability of K1-3, K1-4 and K1-4.5 to induce apoptosis

Up-regulation of FasL was observed in HUVECs treated with K1-3, K1-4 or K1-4.5. To examine if neutralization of the Fas receptor for FasL by anti-Fas antibody had any effect on apoptosis mediated by these molecules, subconfluent HUVECs were pretreated for 1 h with neutralizing antibodies to Fas prior to incubation with null control, K1-3, K1-4 or K1-4.5. Fas receptor neutralization significantly induced the blockage of apoptosis mediated by K1-4 and K1-4.5 (Fig. 4). The blockage routinely achieved 15–25%. Little blockage (< 4%) could be detected in Fas-neutralized HUVECs followed by treatment with K1-3, indicating that Fas receptor plays a more important role in the apoptotic induction by K1-4 and K1-4.5 than K1-3.

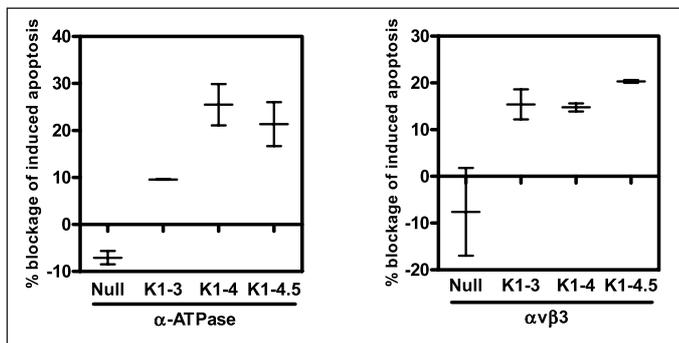


Figure 6: K1-3, K1-4 or K1-4.5 induces apoptosis in endothelial cells through both integrin- $\alpha v \beta 3$ and ATP synthase. Serum-starved HUVECs were treated for 48 h with treatment media containing K1-3, K1-4 or K1-4.5 in presence of anti- α -ATPase (A) or neutralizing $\alpha v \beta 3$ antibodies (B) followed by apoptotic assay. The change of apoptosis in each treatment was expressed as % blockage of apoptosis. Blockage of apoptosis represents the percent decrease of angiostatin-induced apoptotic cells protected by anti-ATPase or integrin antibodies followed by normalization with total apoptotic cells induced by each form of angiostatin in the absence of antibodies. An average, including the range, of two independent experiments for each treatment is shown.

Attenuation of VEGF-induced phosphorylation of AKT by K1-3, K1-4 and K1-4.5

VEGF is a survival factor for endothelial cells and induces phosphorylation of protein kinase B (PKB/AKT) on serine 473 (29). AKT is a regulator of cell survival and apoptosis. We sought to determine by Western blot analysis if any angiostatin molecule affected VEGF-induced AKT activation, which might explain proapoptotic effect of angiostatin. VEGF-treated HUVEC were incubated for 30 min and 24 h with null control, K1-3, K1-4 or K1-4.5, respectively. As shown in Figure 5A and B, K1-3, K1-4 and K1-4.5 differentially attenuated serine 473 phosphorylation of AKT with time compared to the null control. However, two known substrates of AKT, mTOR and GSK3 beta were not attenuated by the treatment of K1-3, K1-4 and K1-4.5 (data not shown). These data indicate that these three forms of angiostatin were capable of mediating their anti-angiogenic effects through reducing the activity of AKT.

Decreased angiostatin-mediated apoptosis by anti- α -ATPase or anti-integrin $\alpha v \beta 3$ antibodies

Angiostatin binds to ATP synthase residing on the surface of endothelial cells and inhibits its enzymatic activity (16, 17). In addition to endothelial cell surface ATP synthase, integrin $\alpha v \beta 3$ has been reported to bind to angiostatin (30). To examine if ATP synthase or integrin $\alpha v \beta 3$ were involved in the induction of apoptosis by null control, K1-3, K1-4 and K1-4.5, the effect of either protein complex on HUVECs was neutralized for 1 h prior to the treatment. Pretreatment of anti- α -ATP synthase antibodies (2.5 μ g/ml) attenuated the ability of all three forms of angiostatin to induce apoptosis while inducing the basal level of apoptosis in cells treated with null control (Fig. 6A). Neutralization of this protein complex achieved highest blockage in K1-4-induced apoptosis (~26%). Neutralization of integrin $\alpha v \beta 3$ also significantly decreased K1-3, K1-4 or K1-4.5-induced apopto-

sis (Fig. 6B). Together, these data suggest that both ATP synthase and integrin $\alpha v \beta 3$ are involved in angiostatin-induced apoptosis.

Differential regulation of gene expression mediated by K1-3, K1-4 and K1-4.5

Since the inhibitory extent of K1-3, K1-4 or K1-4.5 on the proliferating, migratory and tube-forming ability of HUVECs was not completely the same as shown in Figure 1, we then used a gene expression profiling approach to compare if similar or distinct subsets of genes were deregulated by these molecules. Total RNA was isolated for cDNA microarray analysis from HUVECs treated for 4 h with null control, K1-3, K1-4 and K1-4.5. One hundred eighty-nine genes were differentially induced or repressed by two or more average folds in angiostatin-treated cells relative to control using One-way ANOVA ($p < 0.05$). There were 161 induced genes, 6 repressed genes, and 22 differentially regulated genes (see supplementary Tables 2-4 available online at www.thrombosis-online.com). They are assigned into 8 functional groups including growth/proliferation ($n = 62$), inflammation ($n = 24$), apoptosis/survival ($n = 20$), extracellular matrix/adhesion ($n = 14$), migration/cytoskeleton ($n = 14$), energy/metabolism ($n = 8$), protease/anti-protease ($n = 4$), and others ($n = 43$) (Fig. 7A). More than 70% of the 189 deregulated genes fell into the categories of growth/proliferation, inflammation, apoptosis/survival, extracellular matrix/adhesion, and migration/cytoskeleton. For independent confirmation of the chip results, we chose five up-regulated genes, E-selectin (SELE), TIE-1, AXL, PIM-1 and SMAD4 and one down-regulated gene, PPP2R2A for RT-PCR. Consistent with the differential regulation pattern of microarray data, the expression of the former five genes was enhanced (Fig. 7B), whereas that of PPP2R2A was down-regulated by K1-3, K1-4 and K1-4.5 (Fig. 7B). These data indicate that angiostatin molecules mediate anti-angiogenesis partly via gene deregulation at mRNA level.

Increased HL-60 cell adhesion to endothelial cells by K1-3, K1-4 or K4.5

One functional consequence of E-selectin on the endothelial surface can be at the level of leukocyte adhesion (31). To determine if various forms of angiostatin-induced increase of E-selectin played any role in the adhesion of lymphocytes to endothelial cells, subconfluent HUVECs were treated for 16 h with K1-3, K1-4 and K1-4.5 followed by measurement of adhered HL-60 on endothelial monolayers. LPS-treated and EGTA-treated endothelial monolayers, respectively, served as positive and negative controls for lymphocyte adhesion assays. The number of HL-60 cells bound to the monolayer was significantly increased by 5- to 34-fold in the presence of various forms of angiostatin-like molecules (Table 1). The degree of lymphocyte binding to endothelial cells correlated well with the induced level of E-selectin by K1-3, K1-4 and K1-4.5, suggesting that angiostatin-induced E-selection is a functional molecule in mediating adhesion of lymphocytes to endothelial cells.

Discussion

Angiogenesis includes endothelial cell migration, proliferation and tube formation. We demonstrated that three forms of angio-

Table 1: HL-60 adhesion to HUVECs treated with angiostatin.

| Treatment | HL-60 cells bound/cm ² |
|-------------|-----------------------------------|
| 2.5 mM EGTA | 4 ± 2 |
| LPS | 2486 ± 364 |
| Null | 30 ± 7 |
| K1-3 | 1042 ± 36 |
| K1-4 | 368 ± 138 |
| K1-4.5 | 142 ± 45 |

statin, K1-3, K1-4 and K1-4.5, share similar pathways to inhibit angiogenesis. Three apoptotic signaling pathways mediated by p53, FasL and AKT were involved in the anti-angiogenic action of angiostatin. Moreover, at least 134 differentially regulated genes by angiostatin have functions in growth/proliferation, inflammation, apoptosis/survival, migration/cytoskeleton, and extracellular matrix/adhesion.

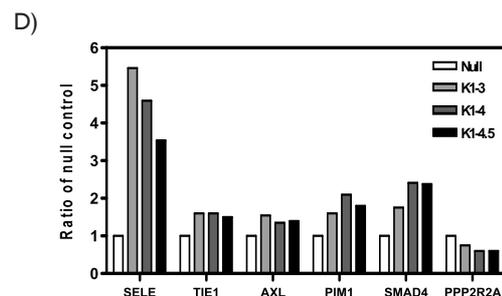
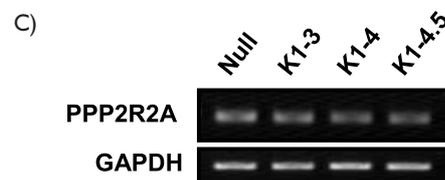
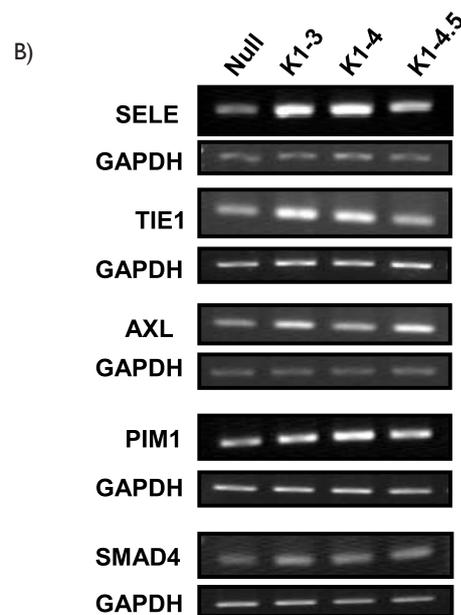
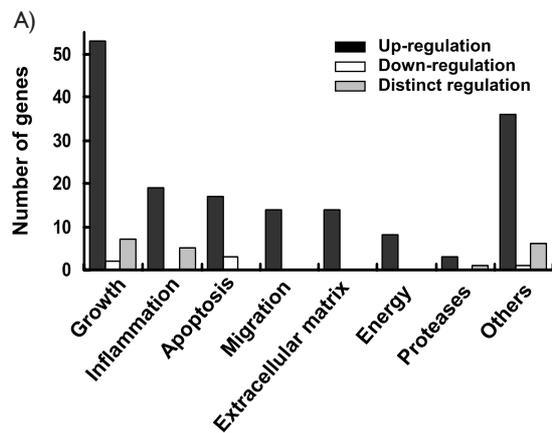
The ability of K1-3 to mediate tube formation was not previously examined until this report. Although K1-3 inhibited endothelial cell proliferation and migration in our studies, we were not able to detect any inhibitory effect of K1-3 on the tubulogenic ability of HUVEC seeded on Matrigel. This process requires both endothelial cell migration and differentiation and is controlled by the balance of proapoptotic and anti-apoptotic signals (32). The inability of K1-3 to block tubulogenesis suggests that either K1-3-insensitive differentiation is a predominant process for tubulogenesis or the lower ability of K1-3 to induce apoptosis may contribute to lower inhibitory effect on tube formation. More studies are needed to address this discrepancy.

Ubiquitously expressed DAPK, a novel family of proapoptotic serine/threonine kinases, participates in many apoptotic systems initiated by IFN- γ , TNF- α , activated Fas, and anoikis (33). Moreover, both extrinsic and intrinsic pathways are involved in DAPK-mediated apoptosis (34). DAPK1 is the prototype of this family and has recently been identified as a transcriptional target of p53 (27). Consistent with DAPK1 being a downstream target of p53, the expression of DAPK1 mRNA together with p53 protein was up-regulated in angiostatin-treated HUVECs. To our knowledge, we are the first to demonstrate the induction of DAPK1 expression by proapoptotic angiostatin. This finding brings a new aspect on the mechanism of anti-angiogenic action mediated by angiostatin molecules. The role of DAPK1 in angiostatin-mediated apoptosis remains to be elucidated.

In addition, K1-3, K1-4 or K1-4.5 attenuated the AKT kinase cascade. Phosphorylation status of two downstream targets

Figure 7: Gene deregulation mediated by K1-3, K1-4 or K1-4.5.

A) The number of genes within each functional category are differentially regulated by angiostatin molecules. Gene annotations were obtained from NCBI database. RT-PCR confirmed angiostatin-induced mRNA expression of five genes including SELE, TIE1, AXL, PIM1 and SMAD4 (B) and angiostatin-reduced mRNA expression of one gene, PPP2R2A (C), using gene-specific primers listed in supplementary Table 1 (available online at www.thrombosis-online.com). D) A quantitative result of mRNA expression ratio of verified gene vs. GAPDH is expressed as a ratio of null control.



of AKT, mammalian target of rapamycin (mTOR) and GSK-3 β , was, however, not affected by any of these angiostatin molecules (data not shown). The AKT substrate(s) involved in angiostatin-mediated inhibition remain(s) to be characterized. Our finding that the anti-angiogenic effect of angiostatin was, in part, mediated by inactivation of AKT and possible distinct downstream effectors underscores the importance of finding the specific target of AKT in angiostatin-treated HUVECs. Recombinant K1–3 from *Pichia pastoris* inhibited migration of skin microvascular endothelial cells toward VEGF and bFGF without altering any signaling pathways, including those mediated by AKT (35). The discrepancy between their result and ours could be due to the cell type difference and/or source of angiostatin.

ATP synthase on the surface of HUVECs is a receptor for K1–3 (16, 36). The binding of K1–4 and K1–4.5 to ATP synthase has, however, never been studied. Using bovine endothelial cells and ectopically expressed integrin in Chinese hamster ovary cells, integrin $\alpha v\beta 3$ can be a predominant receptor for K1–3, K1–4 and K1–5 (30). Recently, K1–5-mediated apoptosis has been shown to be endothelial ATP synthetase-dependent (23). We showed that the presence of anti-integrin $\alpha v\beta 3$ or anti-ATP synthase antibodies attenuated apoptosis induced by K1–3, K1–4 or K1–4.5, indicating a need for integrin $\alpha v\beta 3$ and ATP synthase for angiostatin to propagate the outside-in anti-angiogenic signaling in endothelial cells.

Eighty-two of 189 differentially regulated genes have reported roles in mediating growth/proliferation and apoptosis/survival. The next most deregulated categories by angiostatin are inflammation, migration/cytoskeleton, and extracellular matrix/adhesion. Although only 25% of human genes were surveyed in our custom-made cDNA microarray analysis, we were able to find that angiostatin mediates anti-angiogenesis primarily through deregulation of genes participating in growth/proliferation, inflammation, apoptosis/survival, adhesion/extracellular matrix and migration/cytoskeleton. This finding is not only consistent with known functions of angiostatin in endothelial cells but also a novel anti-inflammatory function of angiostatin.

Expression of E-selectin mRNA was significantly enhanced by K1–3, K1–4 and K1–4.5. This finding is consistent with the

finding that K1–4 up-regulates the expression of E-selectin in proliferating endothelial cells (37). E-selectin, an inducible leukocyte adhesion molecule specifically expressed in endothelial cells, has been implicated in angiogenesis (38, 39). Anti-angiogenic endostatin failed to inhibit bFGF-promoted angiogenesis in E-selection deficient mice. E-selectin significantly enhanced the sensitivity of HUVECs to endostatin, indicating the requirement of E-selectin for the anti-angiogenic effect of endostatin (40). Therefore, the exact role of induced E-selectin by angiostatin molecules remains to be clarified.

In summary, K1–3, K1–4 and K1–4.5, regardless of the kringle number, induced similar pathways to mediate anti-angiogenesis. The pathways include up-regulation of p53 and FasL protein, inactivation of AKT, and gene deregulation. Although the efficacy of K1–3, K1–4, K1–5 in suppression of primary and metastatic tumor growth have been proven in preclinical studies (6, 41–43), the underlying mechanisms for K1–3, K1–4 and K1–4.5 have not been compared until now. Currently, angiostatin K1–3 or the gene encoding this protein is under intense investigation for its anti-tumor effect in the treatment of human cancer. We believe that our work indicates important insights for the anti-angiogenic therapy using angiostatin. Moreover, this study should facilitate the efforts of translating the basic research on the anti-angiogenic action of angiostatin to clinical practice which includes phase I clinical trials with recombinant K1–3 as well as an angiostatin cocktail of direct *in vivo* conversion of plasminogen into K1–4.5 for the treatment of cancer and other angiogenesis-dependent diseases.

Abbreviations

K: kringle; bFGF: basic fibroblast growth factor; VEGF: vascular endothelial growth factor; ATP: adenosine triphosphate; HUVEC: human umbilical vein endothelial cell; BSA: bovine serum albumin; FBS: fetal bovine serum; CM: conditioned medium; PI: propidium iodine; MOI: multiplicities of infection; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; DAPK: death-associated protein kinase; LPS: liposaccharides.

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