Angiostatin K1-3 induces E-selectin via AP1 and Ets1: a mediator for anti-angiogenic action of K1-3

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Summary. *Background*: Angiostatin, a circulating angiogenic inhibitor, is an internal fragment of plasminogen and consists of several isoforms, K1-3 included. We previously showed that K1-3 was the most potent angiostatin to induce E-selectin mRNA expression. The purpose of this study was to identify the mechanism responsible for K1-3-induced E-selectin expression and investigate the role of E-selectin in the anti-angiogenic action of K1-3. *Methods and results*: Quantitative real time RT-PCR and Western blotting analyses confirmed a time-dependent increase of E-selectin mRNA and protein induced by K1-3. Subcellular fractionation and immunofluorescence microscopy showed the co-localization of K1-3-induced E-selectin with caveolin 1 (Cav1) in lipid rafts in which E-selectin may behave as a signaling receptor. Promoter-driven reporter assays and site-directed mutagenesis showed that K1-3 induced E-selectin expression via promoter activation and AP1 and Ets-1 binding sites in the proximal E-selectin promoter were required for E-selectin induction. The *in vivo* binding of both protein complexes to the proximal promoter was confirmed by chromatin immunoprecipitation (ChIP). Although K1-3 induced the activation of ERK1/2 and JNK, only repression of JNK activation attenuated the induction of E-selectin by K1-3. A modulatory role of E-selectin in the anti-angiogenic action of K1-3 was manifested by both overexpression and knockdown of E-selectin followed by cell proliferation assay. *Conclusions*: We show that K1-3 induced E-selectin expression via AP1 and Ets-1 binding to the proximal E-selectin promoter (−356/+1), which was positively mediated by JNK activation. Our findings also demonstrate E-selectin as a novel target for the anti-angiogenic therapy.

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Introduction

E-selectin is an endothelial cell-specific membrane glycoprotein and acts as a receptor that mediates slow rolling and stable arrest of leukocytes on endothelium during inflammation [1,2]. Upon multivalent ligation on endothelial cell surface, E-selectin transduces outside-in signals into the endothelium, leading to changes in intracellular Ca^{2+} concentration, mitogen-activated protein kinase (MAPK) activation, and transcriptional activation of an immediate early response c-*fos* gene [3,4]. A recent study reported that the presence of E-selectin in cholesterol-rich lipid rafts is necessary for its association with and subsequent activation of phospholipase C γ (PLCγ), suggesting that the subcellular localization of E-selectin is related to its signaling function [5].

Overexpression of E-selectin is observed in pathologic angiogenic tissues such as human infantile hemangioma and breast carcinoma, suggesting a pro-angiogenic role of E-selectin [6,7]. Moreover, anti-E-selectin antibodies inhibit capillary-like tube formation *in vitro* [8]. Soluble E-selectin, lacking both the transmembrane and cytoplasmic domains, induces angiogenesis in the rat cornea and stimulates chemotaxis and tube formation of human dermal microvascular endothelial cells via Src and phosphatidylinostiol 3-kinase mediated pathways [9,10]. The proangiogenic role of E-selectin is, however, challenged by two observations. First, angiogenic inhibitors including angiostatin K1-4 and TNP-470 selectively increase E-selectin expression in endothelial cells [11–13]. Second, a study using E-selectin-deficient mice and adenoviral overexpression of E-selectin in human umbilical vein endothelial cells (HUVECs) demonstrated a requirement of E-selectin for the anti-angiogenic effect of endostatin [14]. Although E-selectin expression is strongly related to the events of angiogenesis and anti-angiogenesis, the underlying mechanism by which E-selectin participates in angiogenic modulation remains to be clarified.

Angiostatin is an internal proteolytic fragment of plasminogen and is a naturally existing angiogenic inhibitor. This molecule was initially isolated from mice bearing primary Lewis lung carcinoma. Angiostatin potently suppresses the ability of endothelial cells to undergo proliferation, migration and tube formation, and inhibits murine tumor angiogenesis and metastasis [15,16]. Several angiostatin isoforms, including K1-3, K1-4 and K1-4.5, with differential potency to block angiogenesis *in vitro* and *in vivo* were identified [17–19]. We previously reported that these isoforms mediate angiogenesis via differential effects on gene expression. Consistent with one earlier study by Luo *et al*. [11], the expression of E-selectin mRNA was reproducibly induced by these angiostatin molecules [11,13]. Among them, K1-3 had the highest potency to induce E-selectin mRNA expression. As there are no studies pertaining to the role of E-selectin induction by angiostatin, we thus examined the molecular mechanism responsible for the K1-3-induced expression of E-selectin and investigated whether E-selectin plays any role in angiostatin-mediated inhibition of angiogenesis.

Materials and methods

Materials

Human recombinant VEGF-A was from Pepro Tech (Rocky Hill, NJ, USA). Bovine serum albumin (BSA), gelatin and all chemicals were from Sigma Chemical Co. (St Louis, MO, USA). Reagents for molecular cloning were from Promega (Madison, WI, USA). Reagents for quantitative real time PCR were from Applied Biosystems (Foster City, CA, USA). U0126 and SP600125 were from Calbiochem (San Diego, CA, USA). Pyrolidine dithio-carbamate (PDTC) and BAY 117821 (BAY) were from Tocris Biosciences (Ellisville, MO, USA). Anti-α-tubulin antibody was from Neo Markers (Fremont, CA, USA). Phospho-MAPK and MAPK families were from Cell Signaling Technology (Beverly, MA, USA). Antibodies to E-selectin, Cav1, c-Jun, Ets1 and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Myc antibodies were from Invitrogen (Carlsbad, CA, USA). Rabbit anti-angiostatin K1-3 antiserum was kindly provided by Dr MT Lin at Tzu Chi University (Taiwan).

Cell culture

HUVECs at six passages or less, human lung carcinoma A549, human promyelocytic leukemia HL-60, and human embryonic kidney 293 cells were routinely maintained as previously described [20].

Constructs

pRL-TK and promoterless pGL3-basic vectors were from Promega Biotechnology. Plasmid ELAMlong-pGL3B, kindly provided by Dr Tom McIntyre (University of Colorado, Denver, CO, USA), contains luciferase reporter gene driven by E-selectin promoter. Serially deleted E-selectin promoter constructs (−588/+1, −356/+1, −175/+1, −93/+1 and −83/+1 using transcription start site as +1) were subcloned into pGL3-basic vector by PCR using ELAMlong-pGL3B. Mutated promoter constructs were generated by Quick Change site-directed mutagenesis (Stratagene, La Jolla, CA, USA) using ELAM (−356/+1)-pGL3B as the template. Figure 2 presents a schematic representation of the promoter constructs. Full-length cDNA fragment coding for K1-3 (amino acids 1–352 of plasminogen) or E-selectin was cloned by PCR into adenoviral expression vectors (TaKaRa, Shiga, Japan). Replication-deficient recombinant adenoviruses bearing E-selectin and empty Null adenoviruses (a negative control) were generated and virus titers were determined in 293 cells as previously described [13]. TAM67, a dominant negative c-Jun mutant expression vector, was a gift of Dr Chen BK in the Department of Pharmacology at National Cheng Kung University, Taiwan.

Collection of conditioned media (CM)

K1-3-bearing or Null adenoviral infection of A549 cells at the MOI of 100 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 viral infection was prepared as described [21].

Angiostatin treatment

Subconfluent HUVECs were serum-deprived for 12 h with M199 containing 1% heat-inactivated FBS and 0.1% BSA. Starved HUVECs were treated for the indicated time with the treatment medium composed of 75% CM, 5% heat-inactivated FBS, 5 ng mL^{-1} VEGF-A and 0.1% BSA.

Western blot analysis

Protein concentrations in lysates from HUVECs with or without treatment were measured by Bradford protein assay. Equal amounts of total protein were separated by SDS-PAGE and blotted to a PVDF membrane. The blot was incubated with the indicated primary antibody then with secondary antibody, followed by detection with Renaissance Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA, USA).

Quantitative real-time PCR

Levels of E-selectin were measured by quantitative PCR based on Taqman chemistry using an ABI 7900 HT Sequence Detection System (Applied Biosystems). One microgram of total RNA for each sample, High-Capacity Reverse transcription kit, TaqMan® Gene Expression Assay, FAM™ dye-labeled TaqMan® MGB probe, and TaqMan probe/primer sets for E-selectin (Hs00174057) and GAPDH (Hs99999905), were used for the measurement. The average cycle threshold (Ct) was used to calculate the expression of E-selectin target relative to that of GAPDH reference

using comparative Ct method and the equation: relative expression = $2^{-[Ct(target)-Ct(reference)]}$. Three repeats were performed for each experiment.

HL-60 cell adhesion assay

Confluent HUVECs monolayers were treated for 4 h with 100 ng mL^{-1} liposaccharides (LPS) or the indicated treatment before HL60 adhesion. The adhesion assay was performed as previously described [11]. EGTA at 2.5 mM was used to verify calcium-dependence of selectin-mediated HL60 binding. Bound HL-60 cells were counted in 10 random fields and expressed as mean ± SD per cm^2.

Transient transfection and promoter-driven reporter assay

HUVECs were transfected with the indicated plasmids (E-selectin promoter construct: pRL-TK = 19:1) by using Lipofectamine 2000 (Invitrogen). Four hours post-transfection, cells were incubated for 20 h with treatment medium. Renilla luciferase from pRL-TK serves as an internal control for monitoring transfection efficiency. Luciferase activity in each lysate was measured by Dual-Luciferase assay (Promega) and expressed as relative luciferase unit (RLU).

Lipid raft isolation

HUVECs were treated for 4 h with the indicated CM. Treated cells were lysed and lipid rafts were isolated using raft gradient separation as previously described [22]. Ten 1-mL fractions collected from the top of the gradients were precipitated in 9% trichloroacetic acid then washed with 80% cold acetone before Western blot analysis.

ChIP

We used ChIP assay kits (Upstate Biotechnology Inc., Charlottesville, VA, USA) to examine the *in vivo* binding of AP1 and Ets1 to the E-selectin proximal promoter. Briefly, HUVECs were treated for 4 h with the indicated treatment medium, cross-linked with formaldehyde, lysed and sonicated with the lysis buffer. Chromatin fragments were immunoprecipitated with either 2 μg of control IgG or antibodies to c-Jun and Ets-1, then amplified by nested PCR with three primers specific for E-selectin promoter (Table S1 and Fig. 3C). PCR products were separated by agarose gel electrophoresis.

Fluorescence microscopy

HUVECs grown on cover slips were treated for 4 h with the indicated treatment medium, fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. Fixed cells were blocked for 2 h with 5% FBS then incubated overnight with antibodies to E-selectin or Cav1 in 1% FBS at 4 °C. Following incubation with the fluorescent dye-labeled secondary antibody, fixed cells were visualized under a fluorescence microscope (Olympus BX 60).

Genetic manipulation of E-selectin expression in HUVECs

For E-selectin overexpression, HUVECs were infected for 2 h with empty AdNull or E-selectin-bearing adenoviruses (Ad-SELE) at MOI of 50. Double-stranded oligonucleotide primers coding for short hairpin RNA (shRNA) to knock down E-selectin as described [23] were cloned into pSuper-EGFP vector. HUVECs were transfected for 4 h with control or shRNA-bearing plasmid using Lipofectamine 2000. Genetically manipulated HUVECs were subcultured for proliferation assays at 12-h post-infection or 4-h post-transfection.

Cell proliferation assay

Infected or transfected HUVECs were seeded in quadruplicates at a density of 7000 cells per well in 96-well culture plates, then treated for 48–96 h with indicated CM. Cell proliferation was then measured using MTS kits (Promega).

Statistical analysis

Each experiment was independently repeated three times with similar results. Data were analyzed using Student's *t*-test. Statistical significance was set at $P < 0.05$.

Results

Induction of E-selectin expression and HL60 adhesion by angiostatin K1-3

K1-3 is a potent angiostatin isoform to induce E-selectin expression in HUVECs [11,13]. K1-3 was, therefore, used to address the mechanism whereby angiostatin mediated E-selectin expression. To preserve the native conformation of K1-3 for its anti-angiogenic effect, we collected CM from K1-3 bearing or Null adenoviral infection of A549 cells. K1-3 was only detected in the CM from adenoviral infection bearing K1-3 (Fig. 1A). The level of K1-3 in the CM was estimated to be 20–27 μg mL^{-1}. As the effective range for angiostatin to inhibit angiogenesis is 15–20 μg mL^{-1}, 75% of CM was used to study the anti-angiogenic effect of K1-3 on HUVECs. Quantitative real-time RT-PCR was used to study the induced E-selectin mRNA expression profile by K1-3. Although a basal level of E-selectin mRNA was detected in the Null-CM-treated HUVECs, K1-3 promoted E-selectin mRNA expression as early as 1.5 h then sustained the expression at 4 h post-treatment. By 8 h, E-selectin mRNA expression returned to a level lower than basal expression (Fig. 1B). Western blot analysis confirmed time-dependent expression of E-selectin induced by K1-3, with a peak expression at 4 h post-treatment. By 24 h, there was only residual E-selectin protein expression in the K1-3-treated cells (Fig. 1C). E-selectin mediates leukocyte adhesion on endothelium during inflammation. Pro-inflammatory LPS, a

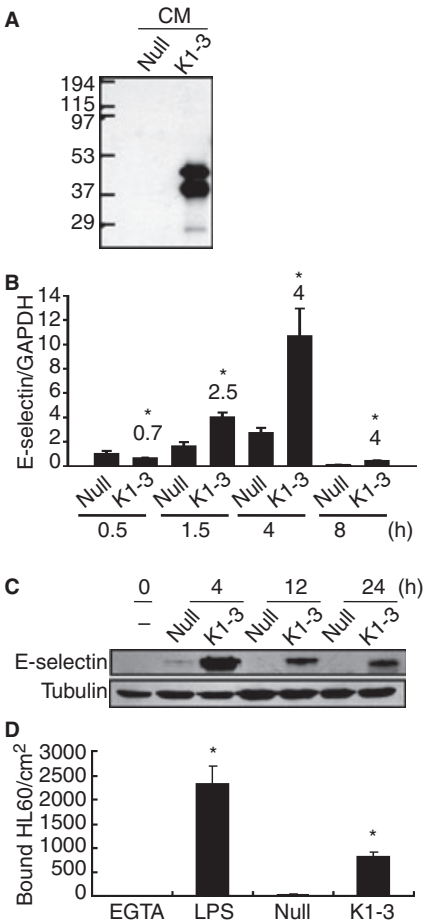

Fig. 1. E-selectin expression and HL60 adhesion induced by K1-3. (A) Western blotting for K1-3 expression in K1-3-CM using antiserum to K1-3. (B) Quantitative real-time PCR analysis of E-selectin mRNA in HUVECs treated for 0.5--8 h with the indicated CM. Numbers at the top of bars represent induction fold of E-selectin expression. (C) E-selectin protein expression in CM-treated cells for 0--24 h. (D) The number of bound HL60 per cm^2 on treated HUVECs for 4 h with indicated CM was measured and expressed as mean ± SD. $*P < 0.05$ vs. Null.

potent inducer of E-selectin, at 100 ng mL^{-1} potently induced leukocyte adhesion (Fig. 1D). Like LPS, K1-3 but not Null-CM induced a significant increase of leukocyte adhesion on HUVECs, indicating that K1-3-induced E-selectin behaves as a functional receptor for leukocyte adhesion on endothelial cells.

Proximal E-selectin promoter required for K1-3-mediated induction of E-selectin transcription

Because K1-3 promoted the expression of E-selectin mRNA at early time points post-treatment, we determined the effect of K1-3 on the E-selectin promoter activity. Following transfection of HUVECs with pGL3B-SELE (−588/+1) and treatment of transfected cells with K1-3 or Null-CM, we determined the relative luciferase activity. Compared with Null-CM-treated cells, K1-3 increased E-selectin promoter (−588/+1) activity by 4–5-fold, demonstrating a direct activating effect of K1-3 on the E-selectin promoter (Fig. 2A). Serially deleted E-selectin promoter segments in the −588/+1 region were used to identify the K1-3-responsive region in the promoter. Peak stimulation was observed in the promoter region between −356 and +1. Deletion of the region between −356 and −175 reduced the response of E-selectin promoter to K1-3 induction. Further removal of the region between −175 and −83 did not only result in very low level of K1-3 responsiveness but also significantly eliminated basal promoter activity (Fig. 2A). Together, K1-3-induced expression of E-selectin mRNA was regulated by the promoter sequence lying between −356 and +1.

We used bioinformatics to identify putative binding sites for TCF1, AP1, NFκB and Ets1 in this region. To study their involvement in K1-3-induced E-selectin expression, we mutated the E-selectin promoter construct at these consensus binding sites (Fig. 2B). TCF1 site mutation had no effect on the expression. NFκB site mutation partially attenuated both the basal and K1-3-inducibility. By contrast, the promoter activity harboring mutated AP1 (mAP1) or Ets1 (mEts1) was significantly lost in both Null and K1-3-CM-treated cells. Combination of both mAP1 and mEts1 sites did not aggravate the response (Fig. 2C). Together, although NFκB binding was

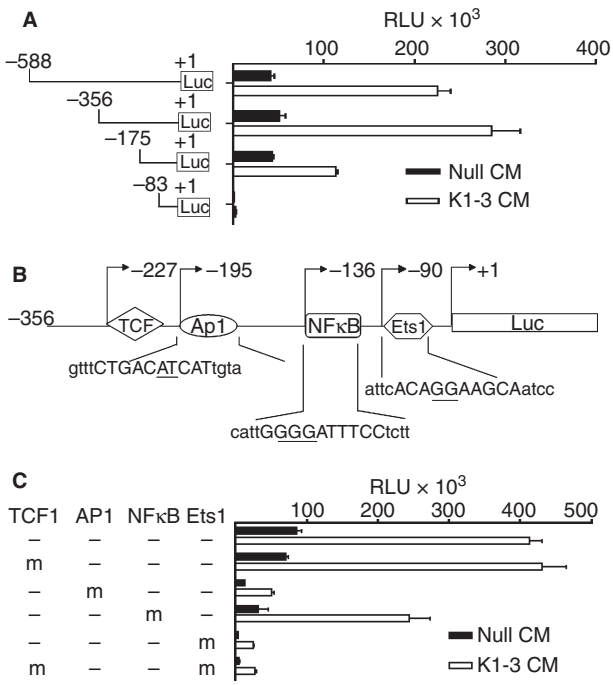

Fig. 2. Activation of the E-selectin promoter by K1-3 via AP1 and Ets-1 binding sites. (A) Plasmids with variable lengths of E-selectin promoter driving the expression of luciferase were transfected into HUVECs. Following CM treatment for 20 h, cells were lysed for measuring the relative luciferase activity. (B) Schematic presentation of E-selectin promoter construct (−356/+1) with putative binding sites for TCF1, AP1, NFκB, Ets-1. (C) HUVECs were transfected with the mutated (m) E-selectin promoter (−356/+1) constructs at individual sites for TCF1, AP1, NFκB or Ets-1 followed by CM treatment. Black bar, CM-Null; white bar, CM-K1-3. K1-3-CM vs. Null-CM, $P < 0.05$

required for the maximal activity, AP1 or Ets1 sites in the proximal E-selectin promoter (−356/+1) were critical elements in both basal and K1-3-induced mRNA expression of E-selectin.

K1-3 induced differential expression and binding of AP1 and Ets1 to the E-selectin promoter

AP-1 complex is composed of Jun and Fos proteins, in which c-Jun is the most potent transactivator [24]. To analyze the temporal relationship of c-Jun and Ets1 with K1-3-induced E-selectin expression, Western blot analysis was used to study the induced profile of these three proteins in K1-3-treated HUVECs. Because K1-3-induced E-selectin expression peaked at 4 h post-treatment, HUVECs were treated for 0.5–4 h with Null or K1-3 medium. A significant induction of E-selectin protein was observed as early as 3 h post-treatment (Fig. 3A). In line with the notion that Ets1 may play a role in K1-3-mediated E-selectin expression, K1-3 increased the expression of Ets1 but not c-Jun at 2 h post-treatment (Fig. 3B). Although c-Jun expression was not increased by K1-3, a slow-moving species of c-Jun-like protein was observed, suggesting a post-translational modification of c-Jun. The transcriptional activity

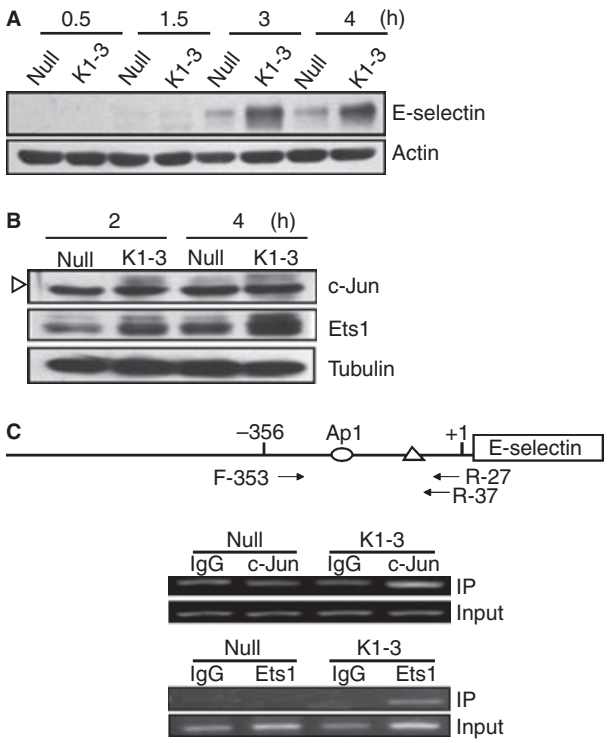

Fig. 3. The induction profiles of E-selectin, c-Jun and Ets1 proteins and *in vivo* binding of c-Jun and Ets1 to their putative sites on the proximal E-selectin promoter by K1-3 Western blot analysis of (A) E-selectin, (B) c-Jun and Ets1 in HUVECs exposed to CM-Null or CM-K1-3 for the indicated time points. Tubulin or actin serves as a loading control. White arrowhead, slow-moving species. (C) Schematic representation of primers flanking the putative Ets1 or AP1 sites for ChIP assay (top panel); following CM treatment, chemically cross-linked and fragmented chromatin was precipitated by antibodies against c-Jun or Ets1 (middle and bottom panels). IgG serves as a negative control.

of c-Jun is regulated by serine phosphorylation [24]. Western blot analysis confirmed that K1-3 increased the phosphorylation level at serine residue 73 of c-Jun protein at 2 h post-treatment (Fig. S1). The time for increasing Ets1 expression and c-Jun phosphorylation was earlier than that for induced E-selectin expression, which further supported the participation of Ets1 and AP1 in K1-3-induced E-selectin expression. ChIP assay (Fig. 3C) and EMSA (data not shown) further confirmed that K1-3 did indeed induce binding of c-Jun or Ets1 proteins to the proximal E-selectin promoter *in vivo* and *in vitro*. Together, these data provide direct evidence for the requirement of AP1 and Ets-1 in K1-3-mediated induction of E-selectin expression.

The localization of K1-3-induced E-selectin in lipid rafts

The presence of E-selectin in Cav1-rich lipid rafts is necessary for E-selectin to propagate its signal [5]. To determine if K1-3-induced E-selectin resided in the lipid rafts, we used a well-established detergent-free sucrose density gradient [22] to preserve the association of molecules in lipid rafts isolated from CM-treated HUVECs. E-selectin was only detected in the buoyant raft fractions 5 and 6, rich in Cav1, from K1-3-treated HUVECs (Fig. 4A). Co-localization of E-selectin and Cav1 was further confirmed in K1-3-treated HUVECs using immunofluorescence microscopy (Fig. 4B). Together, K1-3-induced E-selectin protein was predominantly localized in lipid rafts of HUVECs, suggesting a functional production of E-selectin in K1-3-treated HUVECs.

MAPK family involved in the K1-3-induced expression of E-selectin

The MAPK family (i.e. ERK, JNK and p38MAPK) is known to regulate gene expression [25]. To investigate if any of MAPK signaling pathways was involved in E-selectin induction by K1-3, we examined the activated phosphorylation of ERK1/2, JNK and p38MAPK in CM-treated cells. HUVECs were treated for 30 min with Null or K1-3-CM before lysate harvest for Western blot analysis. K1-3 significantly increased the phosphorylation of JNK and ERK1/2 but not p38MAPK (Fig. 5A). Next we used selective kinase inhibitors to study whether activated phosphorylation of JNK and ERK1/2 was involved in E-selectin activation by K1-3. The inhibitor for JNK (i.e. SP600125) dose-dependently attenuated the K1-3-induced expression of E-selectin (Fig. 5B). By contrast, ERK1/2 inhibition by U0126 had no negative effect on E-selection expression. Instead, U0126 enhanced K1-3-induced E-selectin expression (Fig. 5C). We further confirmed the role of JNK using E-selectin promoter-driven luciferase assay together with pharmacological inhibition of JNK or introduction of c-Jun dominant negative mutant expressing vector (TAM67). Activated JNK inhibition significantly reduced K1-3-induced E-selectin promoter activity (Fig. 5D). Although a typical binding site for NFκB, a known mediator for E-selectin [26], was also present in the proximal E-selectin promoter, an

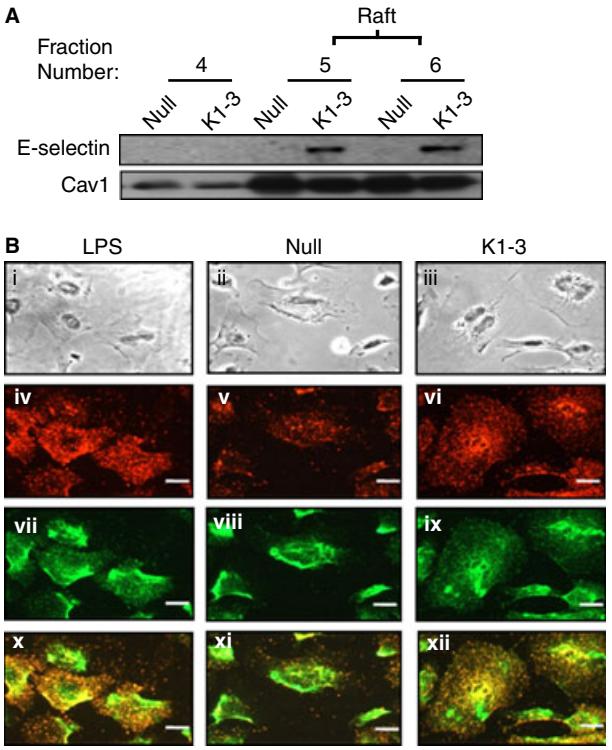

Fig. 4. Co-localization of K1-3-induced E-selectin with Cav1 in lipid rafts. (A) Following treatment, protein lysates were separated by ultracentrifugation on discontinuous sucrose density gradients. Fractions 4, 5 and 6 were analyzed by immunoblotting. Cav1, a lipid raft marker. (B) Immunofluorescence microscopy of K1-3-activated HUVEC shows co-localization of E-selectin and Cav-1. HUVECs were treated for 4 h with 1 μg mL^{-1} LPS (i, iv, vii, x) or Null-CM (ii, v, viii, xi) or K1-3-CM (iii, vi, ix, xii). Red, E-selectin; green, Cav1; yellow, merged E-selectin and Cav1. Scale bar, 10 μm.

effective inhibition of NFκB activation by BAY or PDTC only had marginal effect on the promoter activity (Fig. 5D and Fig. S2). Together, K1-3-mediated induction of E-selectin required the participation of JNK activation.

Effects of E-selectin overexpression or knockdown in the K1-3-mediated endothelial cell proliferation

Angiostatin manifested *in vitro* anti-angiogenic effect in part via inhibition of endothelial cell proliferation. VEGF-A-induced HUVEC proliferation was used as an angiogenic index. To explore the role of E-selectin expression in the anti-angiogenic actin of K1-3, we manipulated E-selectin expression by either adenoviral overexpression or knockdown. The expression level of E-selectin in genetically manipulated cells was first confirmed by Western blot analysis. Although transient induction of E-selectin by K1-3 was not able to chronically enhance E-selectin expression, ectopic E-selectin expression by adenoviral infection persistently enhanced the level of E-selectin in the cells (Fig. 6A and Fig. S3). E-selectin shRNA (SELEi) decreased the K1-3-induced E-selectin expression by 70 to 80% but not tubulin (Fig. 6B and Fig. S4). K1-3 had the highest ability to inhibit VEGF-induced HUVEC proliferation among three

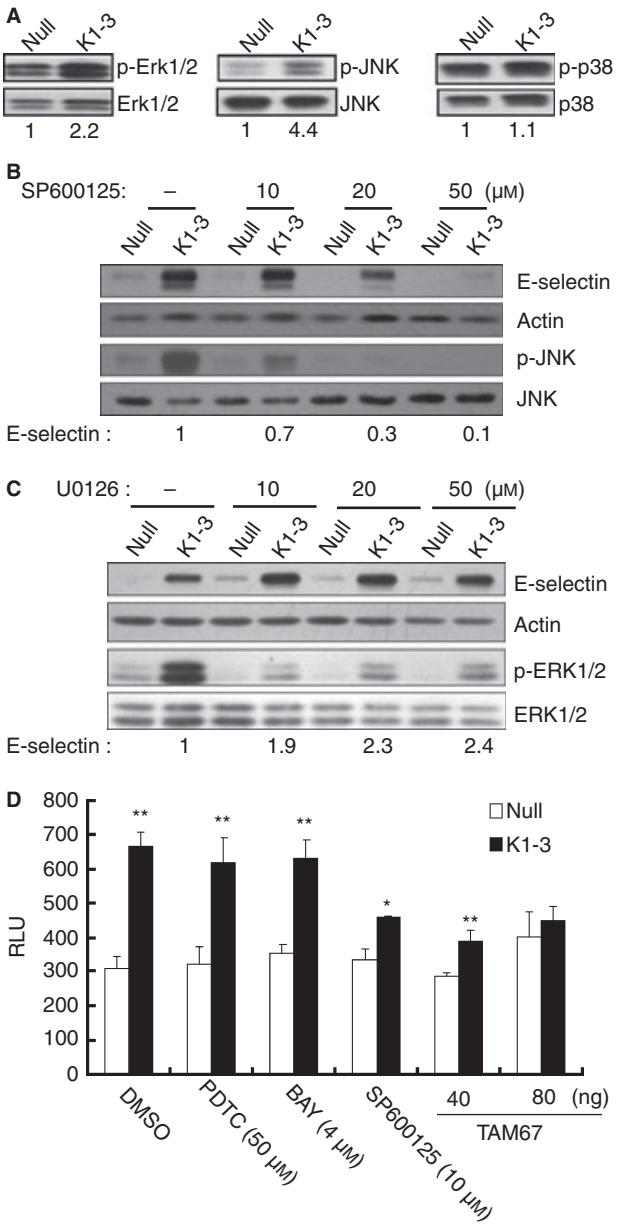

Fig. 5. Requirement of JNK-activated phosphorylation for K1-3-induced expression of E-selectin. (A) Serum-starved HUVECs were exposed to treatment media for 30 min and the lysates were analyzed with Western blot analysis using antibodies against ERK1/2, JNK and p38MAPK and their activated forms (p-ERK1/2, p-JNK and p-p38MAPK). Numbers on the bottom are folds of change. (B, C) Serum-starved cells were treated for 4 h with treatment medium containing the indicated inhibitor before Western blot analysis of E-selectin expression. U0126, inhibitor for ERK1/2 activation; SP600125, JNK inhibitor. Numbers on the bottom indicate the fold change of E-selectin compared with K1-3-treated HUVECs treated with DMSO. (D) Following transfection of E-selectin promoter construct (−356/+1), HUVECs were pretreated with inhibitors then treated with CM followed by luciferase assay. PDTC and BAY, NFκB inhibitors. TAM67, dominant negative c-Jun mutant.

angiostatin isoforms [13]. Consistent with the anti-proliferative effect of K1-3, K1-3 suppressed both VEGF-induced Ad-Null and control cell proliferation (Fig. 6C and 6D). Ectopic E-selectin expression further increased K1-3-mediated inhibition

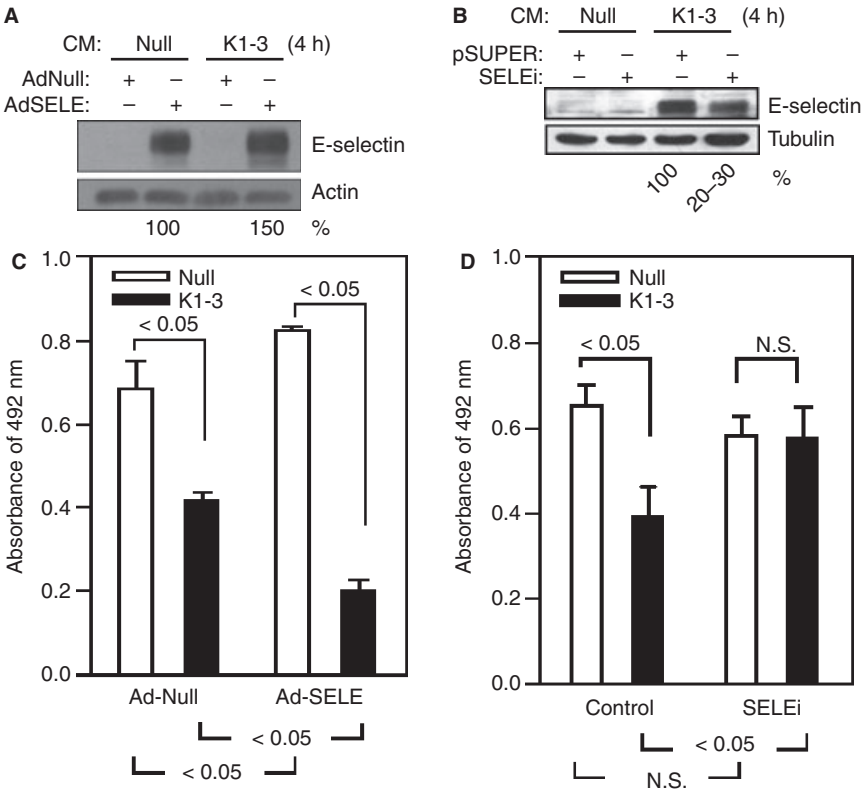

Fig. 6. Alternation of HUVECs sensitivity to the inhibitory effect of K1-3 by manipulation of E-selectin expression. (A, B) Following adenoviral infection for 24 h or transfection of pSUPER control vector or shRNA to E-selectin for 72 h, E-selectin expression in genetically manipulated HUVECs treated for 4 h with CM was confirmed by Western blot analysis. The percentage (%) on the bottom indicates the relative level of E-selectin in the knockdown cells. (C, D) Following E-selectin-bearing adenoviral infection or transient transfection of shRNA-bearing plasmid (SELEi), cells were subjected to cell proliferation assay in the presence of VEGF-A (5 ng mL^{-1}) and CM. White bar, Null-CM; black bar, K1-3-CM; $*P < 0.05$; N.S., not significant.

of HUVEC proliferation (Fig. 6C, $P < 0.05$). By contrast, decreased E-selectin expression by shRNA abrogated K1-3-mediated suppression of HUVEC growth (Fig. 6D). Although alteration of E-selectin expression marginally affected cell proliferation, E-selectin plays a regulatory role in the anti-proliferation effect of K1-3 on HUVECs.

Discussion

We report that angiostatin K1-3 induced the predominant expression of E-selectin in the lipid rafts via promoter activation. The binding of AP1 complex and Ets-1 to the proximal E-selectin promoter was required for the basal expression and K1-3-mediated induction of E-selectin. Putative binding sites for AP-1 and Ets-1 were identified in the proximal promoter of E-selectin (AP1: −195/−186; Ets-1: −90/−81) using promoter-driven reporter assay. The *in vitro* and *in vivo* binding of AP1 complex and Ets1 was confirmed by EMSA and ChIP. We further used pharmacological inhibition to show that inhibition of JNK activation reduced the K1-3-induced expression of E-selectin. Genetic manipulation of E-selectin expression by either overexpression or knockdown indicates a regulatory role of E-selectin in the anti-angiogenic action of K1-3.

AP1 and Ets1 mediate transcription of cytokines and angiogenic factors mainly through their binding to consensus recognition motifs in the regulatory region of these genes [27]. The requirement of AP1 and Ets1 binding in the modulation of K1-3-induced E-selectin expression was manifested at several levels. First, bioinformatics predicted putative binding sites for each complex on the E-selectin proximal promoter (−356/+1). Second, truncation or site-directed mutation of either site abrogated both the basal E-selectin expression and its responsiveness to K1-3. Simultaneous mutations on both sites, nonetheless, had no synergistic effect on the K1-3 response, suggesting that either site alone is sufficient for the induction response of E-selectin. Moreover, transient transfection of endothelial cells with expression vectors encoding either Fos, c-Jun or Ets-1 further enhanced the luciferase activity driven by the promoter (data not shown). Third, we confirmed the *in vitro* and *in vivo* binding of both protein complexes to the proximal E-selectin using EMSA and ChIP. Fourth, Western blot analysis showed that increases in the expression level or post-translational modification of either protein complex occurred before E-selectin induction. In addition to AP1 and Ets1 binding sites, a binding site for NFκB is also present in the proximal promoter region. Although NFκB is the dominant transcription factor for the cytokine-mediated induction of E-selectin [26], mutation of this site only reduced both the basal and induced promoter activity by 50%. Pharmacological inhibition of NFκB only had residual effect on E-selectin promoter activity

(Fig. 5D). Together, AP-1 or Ets-1 plays a more critical role in K1-3-mediated induction of E-selectin than NFκB does.

Cell surface proteins in lipid rafts often participate in signal transmission from cell surface to nucleus [28]. It has been shown that a ligand binding to E-selectin induces not only the cross-linking of E-selectin but also a complex formation of E-selectin with other proteins, leading to ERK1/2 activation [4]. Using immunofluorescence microscopy and lipid raft isolation, we found that the majority of K1-3-induced E-selectin was localized in Cav1-rich lipid rafts, suggesting a functional role of the induced E-selectin. K1-3 potently induced activation of ERK1/2. Inhibition of ERK1/2 activation by U0126, however, had no negative effect on the K1-3-induced expression of E-selectin. Instead, the K1-3-mediated expression of E-selectin was increased by U0126. Our result of K1-3-induced activation of ERK1/2, however, contrasted with one previous study suggesting that angiostatin diminished the activation of ERK1/2 [29]. Whether this discrepant observation was due to the differences in the generation of angiostatin or the type of endothelial cells remains to be characterized.

In addition to ERK1/2 activation, K1-3-induced expression of E-selectin was also preceded by the activated phosphorylation of JNK. JNK is a stress-inducible c-Jun N-terminal kinase and implicated in the apoptotic response of cells exposed to stress. Dual phosphorylation of JNK induces its kinase activity. Phosphorylation of c-Jun by activated JNK protects c-Jun from being ubiquitinated and subsequent degradation [30]. JNK thus plays an important role in the stability and activity of c-Jun. Consistent with the proapoptotic effect of K1-3, we observed not only more than 4-fold increase in the activated phosphorylation of JNK but also increased serine phosphorylation of c-Jun protein in K1-3-treated cells. Pharmacological blockage of JNK activation by SP600125 or expression of c-Jun dominant negative mutant (TAM67) reduced E-selectin induction by K1-3, further supporting the participation of JNK activation in the K1-3-induced expression of E-selectin.

The involvement of E-selectin in angiogenic inhibition is not limited to angiostatin. TNP-470, a fumagillin derivative, is another potent anti-angiogenic compound reported to induce E-selectin expression [12]. Moreover, overexpression of E-selectin rendered human endothelial cells susceptible to the angio-inhibitory effect of endostatin, a natural endogenous angiogenic inhibitor, whereas cells lacking E-selectin lost the ability to respond to endostatin treatment. The same group further demonstrated that the cytoplasmic domain of E-selectin is required for the resistance [14]. In line with a regulatory role of E-selectin in angiogenesis, we also observed that genetic manipulation of E-selectin expression could not only affect the tube forming ability of HUVECs (Fig. S5) but also significantly affected the anti-proliferative ability of K1-3 (Fig. 6C and 6D). Although Yu *et al.* indicated that E-selectin was not required for anti-angiogenic action of angiostatin [14], the dose of angiostatin they used to address the question was 100–1000 ng mL^{-1}, which is more than 18 times lower than the reported effective range to inhibit angiogenesis *in vitro* [31]. Instead, the concentration of K1-3 in the CM we used in this study routinely achieved 20–27 μg mL^{-1}, resulting in a final concentration of 15–20 μg mL^{-1} in the treatment medium. We believe the discrepant observation between this and their study is likely to be due to the dosage variations of angiostatin used in these studies.

In conclusion, our studies show a regulatory role of E-selectin in the angiostatin-mediated anti-angiogenesis. Because E-selectin is one of 189 genes differentially regulated by angiostatin [13], we do not exclude the possibility that additional proteins are also involved in mediating anti-angiogenic action of angiostatin. However, our finding provides a molecular mechanism by which angiostatin K1-3 requires AP-1 or Ets-1 to induce the expression of E-selectin in endothelial cells, and offers a basis for using E-selectin as a target for anti-angiogenic therapy.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sequences.
Fig. S1. K1-3 induced serine[73] phosphorylation of c-Jun.
Fig. S2. TNFα-mediated activation of E-selectin promoter blocked by two selective NFκB inhibitors.
Fig. S3. K1-3 has no chronic stimulatory effect on the increased E-selectin expression in E-selectin overexpressing cells.
Fig. S4. Dose-dependent inhibition of ectopic E-selectin expression by shRNA.
Fig. S5. Modulation of E-selectin expression altered HUVECs tube forming ability on Matrigel.

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