Effect of mutant Endostatin and Kringle 5 fusion protein on Tumor angiogenesis.

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Abstract

Angiogenesis is important for the growth and metastasis of tumors. Endostatin is an endogenous inhibitor of angiogenesis and is shown to inhibit bFGF and VEGF induced signaling in endothelial cells. Recent evidence also indicates that it binds to αv and α5 group of integrins expressed endothelial cells and inhibits downstream signaling. Kringle 5, a potent anti-angiogenic molecule causes apoptosis of endothelial cells by associating with glucose-regulated protein 78 (GRP78). Previous studies from our laboratory have shown that a mutant (P125A) form of Endostatin (derived from collagen type XVIII) and Kringle 5 fragment of plasminogen can inhibit angiogenesis by inducing autophagy. P125A-endostatin and Kringle 5 interfere with distinct signaling pathways in endothelial cells. We hypothesized that a chimeric protein made of P125A-endostatin and Kringle 5 will have better anti-angiogenic activities. To test this hypothesis, we constructed a fusion protein consisting of P125A-endostatin and Kringle 5 (E-K5). Recombinant fusion protein was expressed in yeast and purified. E-K5 was found to inhibit proliferating endothelial cells and effectively blocked tumor induced angiogenesis. Anti-proliferative activity of E-K5 was linked to VEGF receptor and αvβ3 integrin-mediated signaling pathways. These studies establish the therapeutic potential of E-K5 as a potent anti-angiogenic molecule.

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Introduction

Cancer is a leading cause of death worldwide and it is predicted that the number of new cancer cases will rise from 14 million (2012) to 22 million in the next 20 years. In the United States the overall cancer death rate has declined since 1990, showing that remarkable progress has been made in the development of strategies for early detection and treatment. However, cancer is far from being eradicated. Recurrence and resistance development following the initial treatment limit the survival of cancer patients. Thus there is a need for new secondary line of drugs to treat recurrent disease (1). Recent technological revolutions in whole genome sequencing and proteomics have identified several dysregulated molecular signaling pathways in cancer cells. Studies have shown the therapeutic importance of targeting the genetic changes accrued by cancer cells that drive the induction, progression and invasion of tumor (2). Understanding the consequence of these altered pathways provides unique opportunities to develop novel drugs to treat cancer.

Ovarian cancer ranks fifth in the cancer deaths among women. The absence of early detection has caused a decline in the rate at which women are diagnosed with ovarian cancer in the past 20 years, making it the deadliest gynecological malignancy. The American Cancer Society estimates that in 2016, about 22,280 women will be diagnosed with ovarian cancer in the United States. Epithelial ovarian cancer arises either from the ovaries or fallopian tube. Ovarian cancers

are confined to the site of origin at early stage (Stage I) and metastasize into the peritoneum during tumor progression.

Tumor Microenvironment and Angiogenesis

It is now well established that cancer cells modify and remodel their surrounding to support their survival. Tumor and its micro environment (TME) is a complex interface between cancer cells, immune cells, vascular endothelium, stromal cells and extracellular matrix components (ECM). Crosstalk between tumor cells and tumor infiltrating cells facilitates tumor invasion and progression, which is characterized by alterations in metabolic pathways, increased angiogenesis and evasion of immune surveillance and epigenetic regulation of survival pathways. The two hallmarks of cancer, angiogenesis and metastasis are highly dependent on TME (1). Angiogenesis, the formation of new blood vessels from preexisting vasculature is important for the growth and metastasis of tumors. Inhibition of angiogenesis therefore is expected to have profound effects on tumor progression.

Angiogenesis is absolutely necessary during development. Inhibition of any of the angiogenic pathways during development is lethal. In adults, angiogenesis is necessary for tissue remodeling such as wound healing, menstrual cycle, pregnancy and collateral vessel growth after an ischemic injury. Physiological angiogenesis is tightly regulated by maintaining a balance between proangiogenic factors including VEGF, FGF, TGF-α, PDGF, IL-8, angiopoietin-1 and

endogenous angiogenesis inhibitors such as angiostatin, endostatin, thrombospodin, plasminogen activator inhibitor-1. Disruption of this homeostatic mechanism will result in vascular pathology. Tumor cells highjack the same physiological angiogenic cues to promote their growth and spread to distant secondary sites. A fundamental signal for angiogenesis is the availability of oxygen. When tumors surpass their nutrient and oxygen supply, a hypoxic environment is created. Under hypoxic stress, tumor cells secrete a number of pro-angiogenic growth factors. The molecular mechanism of pro-angiogenic growth factor expression is triggered by the stabilization of a transcription factor, Hypoxia induced factor-1 (HIF-1) and Hypoxia induced factor-2 (HIF-2). HIF is a heterodimeric protein made of an alpha subunit and beta subunit. Beta subunit of HIF is constitutively expressed. Under normoxic conditions, HIF-1 alpha is maintained very low levels by polyubiquitination and proteasomal degradation. Two proline residues in HIF-1 and HIF-2 alpha are hydroxylated by prolylhydroxylases (PHD) under normoxia. Hydroxylated HIF-1/2 alpha subunits are then recognized by VHL. A scaffolding protein, CUL2, then assembles E3 ligases such as Elongin and RBX1. Polyubiquitinated protein is degraded by proteasomes. PHD enzymes function as oxygen sensing molecular switches and their activity is dependent on oxygen, iron, ascorbic acid and alpha-ketoglutarate, a product of the tricarboxylic cycle of glucose metabolism. Hypoxia inhibits PHD enzymes and thus prevents proteasomal degradation leading to the accumulation

of HIF-1/HIF-2 alpha. HIFs then dimerize with HIF-1 beta subunit, binds to HIF-responsive elements (HRE) and induce expression of angiogenic factors such as Vascular endothelial growth factor (VEGF) (3). VEGF signaling predominantly drives sprouting of angiogenesis and is also involved in recruiting progenitor endothelial cells from the bone marrow to growing vasculature. In addition, hypoxia upregulates the expression of matrix metalloproteinases (MMPs) and activates other proteases. This results in the degradation of the basement membrane surrounding the tumor, driving tissue remodeling, tumor progression and metastasis (4). Interestingly, matrix degradation also releases anti-angiogenic fragments such as endostatin from collagen type XVIII. Normal tissues maintain a strict balance between pro and anti-angiogenic factors. Homeostatic balance is however disrupted in the TME in favor of sustained pro-angiogenic signals.

As a result the blood vessels generated in the tumors are often abnormal. They are leaky and tortuous leading to high interstitial pressure. Increased fluid pressure collapses vessel wall and slows down blood flow. Abnormal vessels in combination with reduced blood flow in tumor vessels make drug delivery to tumor tissues as a major problem (5). Additionally, continuous remodeling of the vasculature due to TME, vessel permeability differs in various areas of the tumor. Consequently, hypoxic areas with limited nutrient supply are created. Persistent hypoxia increases intra-tumor heterogeneity, induces resistance to chemotherapy and provides a niche for cancer initiating stem cells. Thus, tumor

hypoxia is intricately linked to progression, metastasis and treatment resistance.

Table i.

Major pathways involved in Tumor angiogenesis

Signaling Molecules	**Implicated role in Tumor Angiogenesis**
VEGF- VEGFR-2/ VEGF-VEGFR-1	Proliferation and sprouting of endothelial cells, cell survival and suppress apoptosis, inhibition of NF-κB activation and defective immune response, sustaining angiogenesis.
Dll4- Notch	Increased expression of Dll4 in tumor vasculature blockade results in overgrowth and abnormal vasculature – suppress tumor growth.
αν Integrins	Endothelial cell protection from apoptosis, amplification of angiogenic signals by complexing with growth factor receptors, production of MMP-2 and driving metastasis.

PDGF-BB- PDGFR-β	Induction of endothelial cell proliferation and migration, increased oxygen perfusion (6).

Role of Integrins in Angiogenesis

Since ECM remodeling supports endothelial cell proliferation and invasion, it has a major role in regulating angiogenesis. Proteolytic changes in the ECM, in response to growth factor stimulation are capable of exposing sites crucial for angiogenesis regulators like integrins. For instance, proteolytic cleavage of type IV collagen exposed a cryptic site for αvβ3 integrin binding (7). Integrins are the family of cell adhesion molecules that are present on endothelial cells that bridge cell – ECM attachment triggering signals that result in increased migration and motility. Quiescent endothelial cells express different combinations of the α and β subunits including α1β1, α2β1, α3β1, α5β1 and αvβ5. Interestingly, αvβ3, a receptor for vitronectin is not generally expressed in quiescent blood vessels, but is dramatically upregulated in angiogenic blood vessels (8). The integrin αvβ3 has a 125kDa αv subunit and a 105kDa β3 subunit and has the ability to bind to numerous ECM ligands containing an Arg-Gly-Asp (RGD) peptide motif. The importance of αvβ3 in tumor angiogenesis, led to the development of monoclonal antibodies against αvβ3 and cyclic RGD peptide mimetics that inhibit growth and survival of endothelial cells (9). Further proving this concept, LM609, an

antibody to αvβ3 has shown to efficiently target angiogenesis in several pre-clinical models (10). Intetumumab, a humanized antibody against αv integrins-αvβ3 and αvβ5 is currently in phase II trials for the treatment of melanoma (11) and metastatic, castration-resistant prostate cancer (12). These studies suggest a coordinated program of ECM degradation, remodeling and integrin expression are important for sustained angiogenesis in TME.

VEGF- VEGF-R signaling in angiogenesis

The VEGF family of growth factors comprises of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PIGF. Of the various growth factors recruited into the TME, Vascular endothelial growth factor (VEGF)/ VEGF-A expression has been shown to directly correlate with malignant progression. Higher VEGF levels in patients with cancer (<12.5–>900 pg/mL) in comparison to healthy subjects (<12.5–49 pg/mL) could predict the adverse outcome in metastatic colorectal cancer, lung cancer and renal cell carcinoma (13). VEGF-A levels are regulated by numerous mechanisms. For instance, hypoxia is a key driver of angiogenesis, which results in the induction of Hypoxia inducible factor 1α (HIF-1α) stabilization. HIF-1α is a major transcription factor that binds to hypoxia response elements, initiating transcription of the VEGF gene (14). Secondly, cytokine induced activation of tyrosine kinase receptors including, Epidermal growth factor receptor (EGFR), Human epidermal growth factor receptor 2

(HER2) (15), Fibroblast growth factor receptors (FGFR) (16) and Insulin like growth factor 1 receptor (IGF) (17), also induce VEGF-A expression. VEGF-A mediates its effects by binding to and activating VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), promoting endothelial cell proliferation and migration, vascular permeability, gene expression and thus playing a central role in promoting tumor angiogenesis and progression. VEGF-A has a higher affinity to VEGFR-1, but the major contribution to the angiogenic stimulation involves VEGFR-2, because the tyrosine kinase activity of VEGFR-2 is 10 fold stronger than VEGFR-1 (18). Normal blood vessels show negligible expression of VEGFR-2 whereas the blood vessels supplying tumors have increased expression of VEGFR-2, providing a selective advantage for targeting angiogenic vessels and preventing metastatic spread.

Numerous studies have implicated the association of the VEGF-VEGFR system with integrins and have concluded the cooperation and cross-talk between these signaling systems. Interaction of integrins with the ECM proteins triggers outside-in signaling which leads to activation of kinases such as Focal adhesion kinases (FAK), MAP kinases and changes in calcium flux. Since αvβ3 is one of the key regulators of endothelial functions and is significantly upregulated in tumor endothelium, cooperation between αvβ3 and VEGFR-2 has been actively studied. Endothelial cell adhesion on a vitronectin matrix (αvβ3 ligand), enhances the phosphorylation of VEGFR-2 and blocking antibodies to both αv /β3

inhibited increased phosphorylation (19). In addition, VEGF-A binding to VEGFR-2 results in the association with only the β3 subunit of αvβ3 but not with β1 or β5 integrins on ECs (20). Furthermore, involvement of c-Src and FAK cooperating between VEGFR-2 and αvβ3 signaling has been shown in activated endothelial cells (20) (21). In conclusion, reciprocal signaling between αvβ3 and VEGFR-2 results in integrin activation and phosphorylation by signaling mediators (c-Src and FAK) (22). Thus, targeting this feed forward loop presents an attractive strategy for anti-angiogenic therapy.

Targeting Angiogenesis- Limiting the blood supply

Targeting angiogenesis will restrict the supply of nutrients and oxygen to tumors and inhibit tumor progression. The significance of the VEGF/VEGFR system prompted the development of several agents targeting this system. The first clinically used angiogenesis inhibitor, Bevacizumab (Avastin), is a humanized monoclonal anti-VEGF antibody. Treatment of cancer patients with Bevacizumab along with chemotherapy improved progression free survival (PFS) in majority of the cancers including Non small cell lung cancer (NSCLC), glioblastoma, ovarian cancer and metastatic colorectal cancer (23). Despite the early successes of anti-angiogenic therapy, most of the treated patients develop resistance intrinsic and acquired drug resistance. For instance, pancreatic tumors are notoriously less vascularized with a dense stroma. Bevacizumab treatment is not effective in

treating advanced pancreatic cancer patients (24). Another barrier for success of anti-angiogenic therapies is the upregulation of alternate pro-angiogenic factors including bFGF, HGF, and PIGF (25). Hence, the development of a broad-spectrum angiogenesis inhibitor, having multiple targets is an attractive strategy for the successful treatment of cancer. Endogenous angiogenesis inhibitors are naturally produced in the body to maintain the angiogenic balance and therefore are least toxic. Many of them are derived from the proteolytic fragmentation of ECM components. To date, at least 30 different endogenous small molecules and peptides have been identified that inhibit angiogenesis (26). Some of the important endogenous inhibitors of angiogenesis are listed in Table 2.

Table ii.

Endogenous inhibitors of angiogenesis

Inhibitors	**Identified Receptors/targets**
Angiopoietin 2	Angiopoietin 1 receptor
Angiostatin (Kringle 1-4)	Angiomotin, αvβ3, ATP synthase,
Canstatin	Angiopoietin 1, αvβ3, αvβ5
Endorepllin	α2β1, VEGFR2
Endostatin	αv and α5 integrins, VEGFR2, glypican 1, MMP-2, -β-catenin, HSPG

Kringle 5	GRP-78, VDAC
16kDa Prolactin	Receptor unknown, upregulates PAI-1
Prothrombin Kringle 2	αvβ3
Thrombospondin 1	CD36
Tumstatin	αvβ3, α3β1
VEG1	DR3, Dcr3

Endostatin is a 20kDa proteolytic fragment of the non-collagenous C-terminal domain of collagen XVIII. It was first purified and characterized from murine hemangioendothelioma tumor cells (27). Numerous studies have shown that endostatin inhibits endothelial cell proliferation and migration in vitro and tumor angiogenesis in vivo (27) (28). Elevating the serum levels of endostatin by genetic overexpression suppressed tumor growth. Since endostatin is known to have a broad-spectrum of targets, it exerts its anti-angiogenic activity by acting through multiple signaling pathways. A molecular analyses of endostatin treated endothelial cells showed alterations in ~ 12% of the genome, targeting several regulatory genes involved in angiogenesis (29). Endostatin directly binds to VEGFR-2 and inhibits the VEGF-induced tyrosine phosphorylation of VEGFR-2 and activation of downstream signaling (30). Interestingly, Eriksson et al. showed that endostatin inhibits VEGF/FGF induced migration of endothelial cells without affecting the phosphorylation of p44/42 MAPK, p38 MAPK or PLC- Γ (31).

Binding of endostatin to the αvβ3, α5β1 and α2β1 integrin family of receptors results in disruption of cell-matrix interactions and activation of apoptotic cascade (32) (33) (34). In spite of its diverse and complex mechanism of action in the inhibition of angiogenesis and tumor growth, it is reported that endostatin would have an increased biological activity when delivered as a dimer or through genetic modification (35). Previous studies from out laboratory have shown that, human endostatin containing a point mutation at position 125 (P125A) had higher tumor homing, better inhibition of angiogenesis and tumor growth compared to native endostatin by downregulating VEGF and Ang 1 mRNA levels (36). Furthermore, targeting endostatin by the addition of NGR motif, a peptide recognized by APN on the tumor vasculature, increased binding to endothelial cells an improved anti-angiogenic activity (37). These studies proved that understanding the structure and function of endostatin will help in improving its binding to endothelial cells, resulting in increased tumor localization and enhanced anti-angiogenic potency.

Kringle domains of plasminogen are one of the most well studied endogenous angiogenesis inhibitor. Angiostatin (Kringle 1-4) a 38kDa plasminogen fragment was shown to inhibit the growth of metastases in Lewis lung carcinoma tumor model (38). Interestingly, Kringle 5 of plasminogen inhibited bFGF induced endothelial cell proliferation better than angiostatin and had more potent anti-angiogenic activity (39). Subsequent studies show that it inhibits endothelial cell activity by associating with glucose- regulated protein 78 (GRP78) / voltage

dependent anion channel (VDAC1) and activating caspases (40) (41). Recently K5 was found to down-regulates VEGF expression by suppressing the HIF-1α pathway. The exact mechanism for its anti-angiogenic activity still is a matter of interest in the research field, as it is known to exert its effect on cell migration (42), interfering with autophagic survival response and activating apoptosis pathway (43). Though endostatin has the broadest repertoire of targets and showed effective inhibition of tumor angiogenesis and growth *in vivo*, it did not show consistent anti-tumor activity in clinical trials (44). Over the years, some of these endogenous inhibitors have reached clinical trials but their long-term therapeutic efficacy seemed questionable. Endostar (recombinant endostatin) is the only approved anti- angiogenic agent in China. Endostar in combination with chemotherapy is currently under clinical trials for the treatment of Non-small cell lung cancer and Peripheral T-cell Lymphoma. However, endostatin efficacy is still limited by bioavailability and potency. There is an unmet need to prepare potent endostatin molecules with improved pharmacokinetic and pharmacodynamics properties. Current studies are directed to achieving these goals by genetically engineering a fusion protein to improve the biological activity of endostatin.

Hypothesis

Genetic fusion of two anti-angiogenic proteins that inhibit distinct signaling pathways in endothelial cells will improve the therapeutic efficacy of angiogenesis inhibition.

Specific Aims/objective

A novel fusion protein E-K5, was prepared by genetically splicing P125A endostatin and Kringle 5 fragment of human plasminogen. It is proposed to determine the effect of the fusion protein on human endothelial cells and angiogenesis. Another objective of this study is to understand the molecular pathways affected by the fusion protein to unravel additional targets for drug development.

Results

Purification of fusion protein E-K5

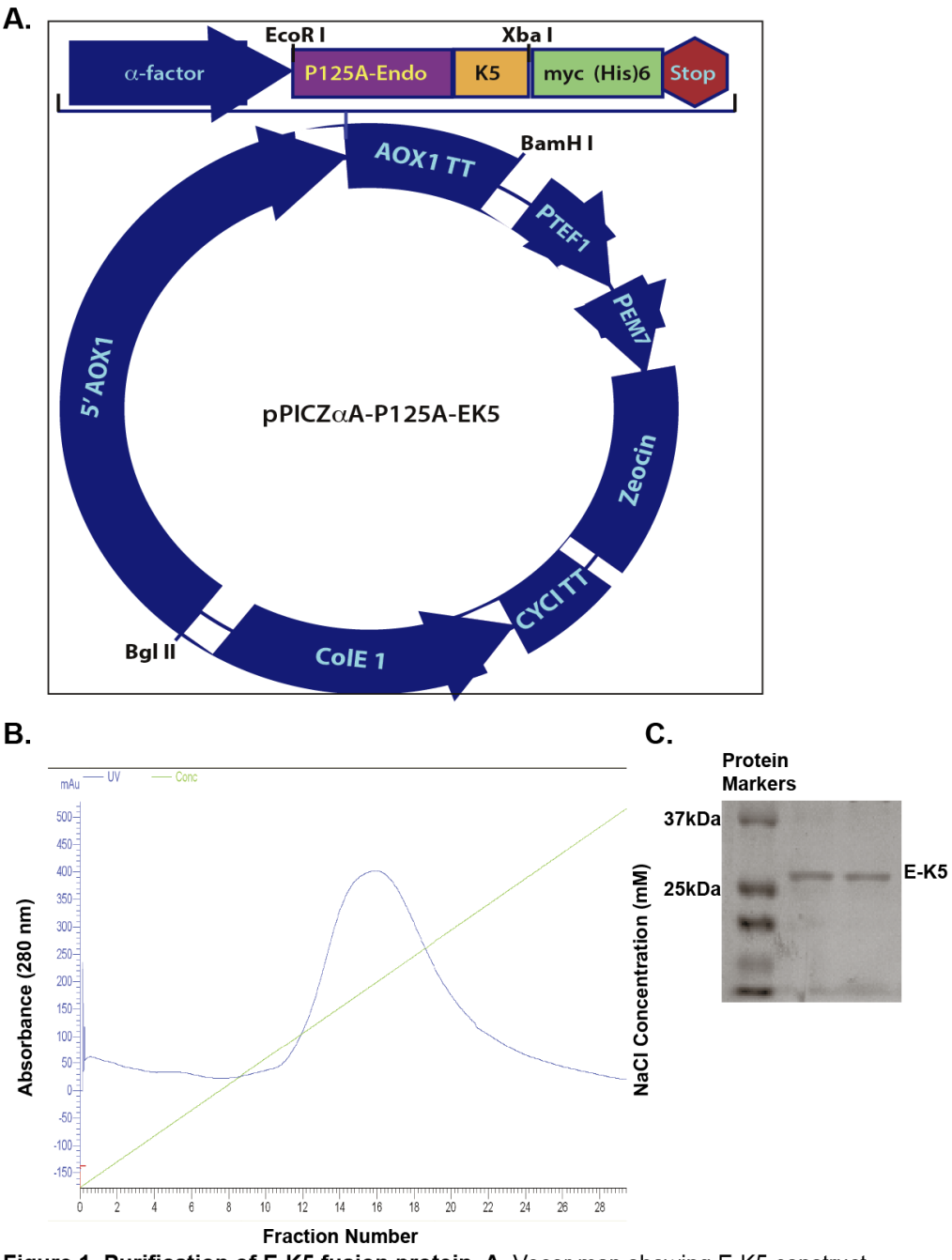


Figure 1. Purification of E-K5 fusion protein. A. Vecor map showing E-K5 construct. E-K5 fusion protein was purified using HiTrap Heparin HP Columns. **B.** Elution profile of E-K5. **C.** Comassie staining showing E-K5 resolved by SDS PAGE.

E-K5 inhibits cell attachment to fibronectin (FN) and vitronectin (VN) matrix.

Endothelial cell attachment to extracellular matrix is mediated through integrins on the cell surface, promoting cell survival and migration. Since previous literature has shown that endostatin interacts with α5 and αv group of integrins (32), we studied the attachment of HUVEC-I in the presence of E-K5 to fibronectin and vitronectin-ligands to α5β1 and αvβ3 respectively. Real time measurement was carried out to study attachment of HUVEC-I on FN (10ug/ml) and VN (5 ug/ml) coated wells. The initial phase of the graph shows that the cells sediment and after 2 hours start attaching and spreading which is measured as impedance (Cell index). In presence of E-K5, cell attachment to fibronectin was decreased within 2 hours and a further decline was seen at 6 hours (Figure 2A). Cell attachment and spreading to vitronectin-coated wells also was significantly inhibited. Treatment with E-K5 showed better inhibition of endothelial cell attachment when compared to P125A (Figure 2 B).

E-K5 directly binds to α5β1 and αvβ3 integrins on HUVEC-I

Since E-K5 inhibited the attachment of HUVEC- I to fibronectin and vitronectin, we examined if, fibronectin receptor α5β1 and vitronectin receptor αvβ3 were involved in cell attachment. Further experiments demonstrated that the binding partners for E-K5 on HUVEC-I were the α5β1 and αvβ3 integrins. As shown

in Figure 2C, control wells coated with E-K5 showed 100 % attachment to HUVEC-I. To investigate the involvement of α5β1 and αvβ3, anti- integrin antibodies were used to block the specific interaction. Monoclonal antibody against α5β1 JBS5 treatment of endothelial cells (EC) inhibited 80 % of EC attachment to immobilized E-K5 as measured by the fluorescence intensity. LM609 against αvβ3 also inhibited attachment to the same extent confirming that, HUVEC-I attachment to immobilized E-K5 directly involves integrins α5β1 and αvβ3. Thus E-K5 inhibits cell attachment to ECM, through fibronectin receptor α5β1 and vitronectin receptor αvβ3.

As mentioned previously, the role of integrin αvβ3 is complex in angiogenesis involving crosstalk with VEGFR-2 and thus to further validate the involvement of αvβ3, real time cell attachment assays were carried out using the RTCA DP Instrument. HUVEC-I were pre-incubated for 30 min with control IgG or antibody to αvβ3 followed by treatment with E-K5 (20ug/ml). There was a significant inhibition of attachment and spreading of cells incubated with E-K5 alone or control IgG or Ab +E-K5 to Vitronectin coated E-plate compared to control wells validating the results shown in Figure 2B. HUVEC-I incubated with an antibody to αvβ3 also showed significantly greater inhibition of cell attachment. Interestingly, cells incubated with both antibody to αvβ3 showed a 10-fold greater inhibition of cell attachment compared to αvβ3 alone and 20- fold decrease in cell attachment when cells were treated with E-K5 alone (Figure 2D). Thus, E-

K5 binds to αv and α5 integrins on HUVEC-I and inhibits their attachment to ECM and this effect is further enhanced by an antibody to αvβ3. These results suggest that, addition of E-K5 treatment to therapies targeting αvβ3 such as Intetumumab, can further increase inhibition of endothelial cells and tumor angiogenesis.

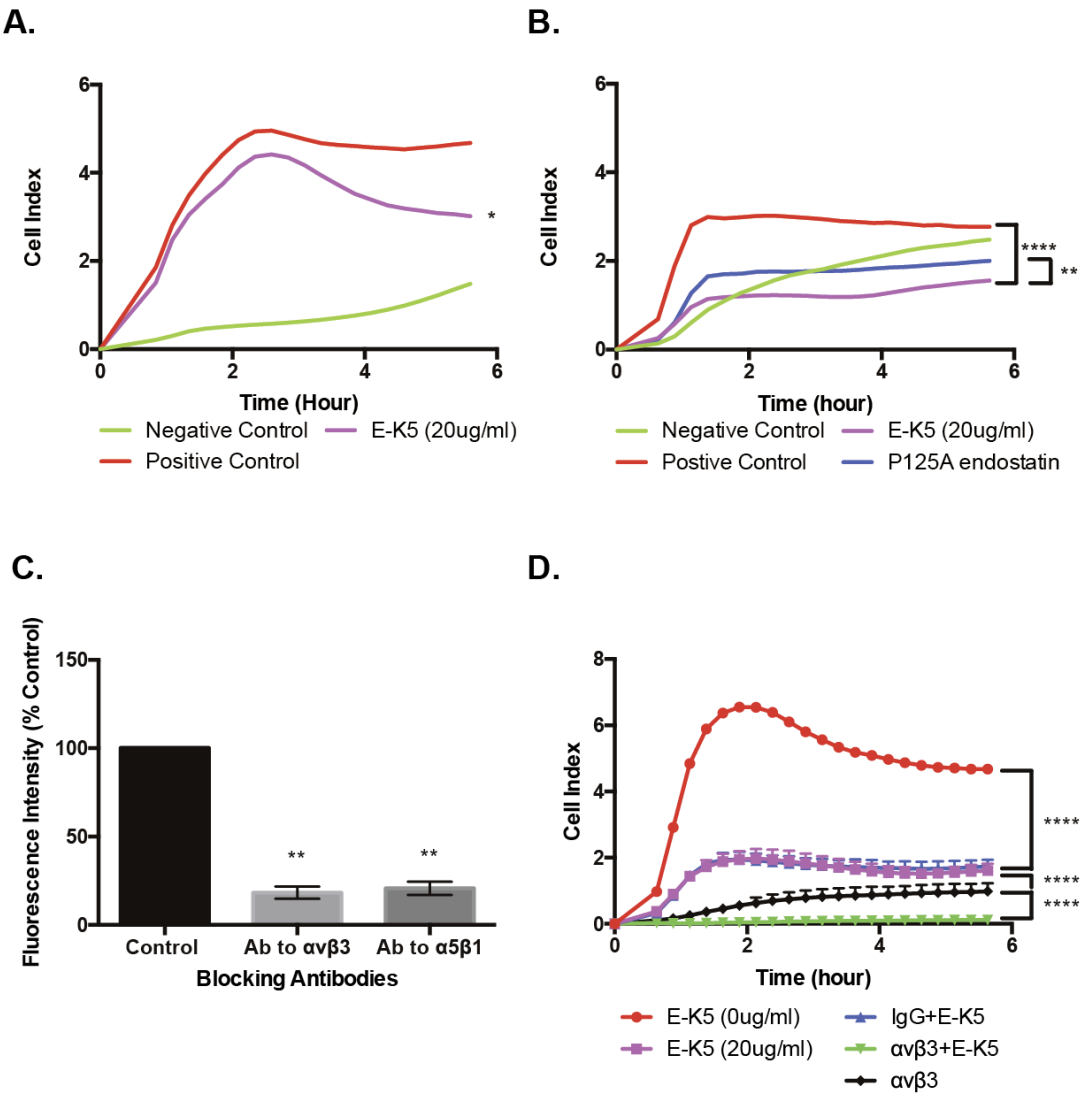


Figure 2. Effect of E-K5 on HUVEC-I attachment. A and B. Real time cell attachment measurement of HUVEC-I on FN (10ug/ml) and VN (5ug/ml), respectively. E-K5 treated cells showed decrease in attachment and spreading after 2 hours (n=2). **C.** Direct Binding of prelabelled HUVEC-I with cell tracker blue to immobilized E-K5 was determined by measuring the fluorescence intensity of bound cells. Wells incubated with antibodies to αVβ3 and α5β1.showed inhibition of HUVEC-I interaction to the respective integrin receptors. Values represent mean of two independent experiments

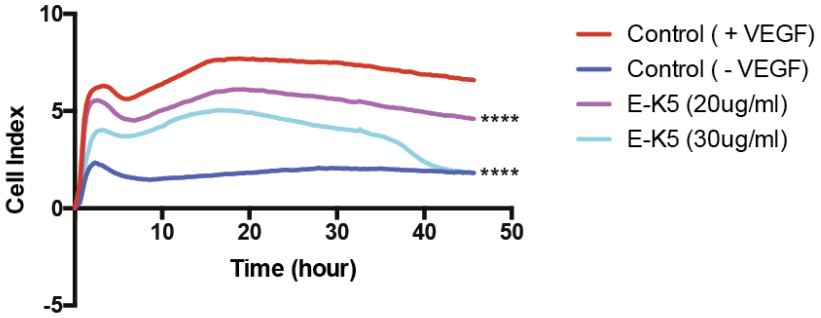
D. Real time cell attachment measurements showing effect of E-K5 in the presence of antibody to αVβ3 (n=3). Data are expressed as means ± SD. Statistical analyses were performed using GraphPad Prism® 6. Differences in mean values between the two groups were analyzed using the two-tailed Student's t-test.

*P ≤ 0.05 **P ≤ 0.01 ***P ≤ 0.001 ****P ≤ 0.0001 compared to control.

E-K5 inhibits VEGF-A induced endothelial cell proliferation

Endothelial cell proliferation is important for tumor angiogenesis. Previous studies have shown that endostatin inhibits bFGF-induced proliferation of BCE cells (27) and VEGF-A induced proliferation of CVEC (45). Similarly, recombinant Kringle 5 (rK5) has been found to inhibit VEGF-A induced proliferation of HUVEC (43). To investigate whether the fusion protein inhibited VEGF-A induced cell proliferation, real time measurement of cell proliferation was recorded as described in *Materials and Methods*. Since E-K5 inhibits cell attachment and spreading, E-K5 inhibited endothelial cell index (impedance) in as less as ~3 hours. HUVEC-I proliferated in presence of VEGF-A (100ng/ml) at ~12 hours (Fig. 3A). Cells treated with E-K5 showed a steady decline in cell index after 20 hours continuing up to 48 hours. To further confirm these results, we studied the effect of E-K5 on primary endothelial cells (HUVECS). These studies showed a similar concentration dependent inhibition of VEGF-A induced EC proliferation (Figure 3B).

A.



B.

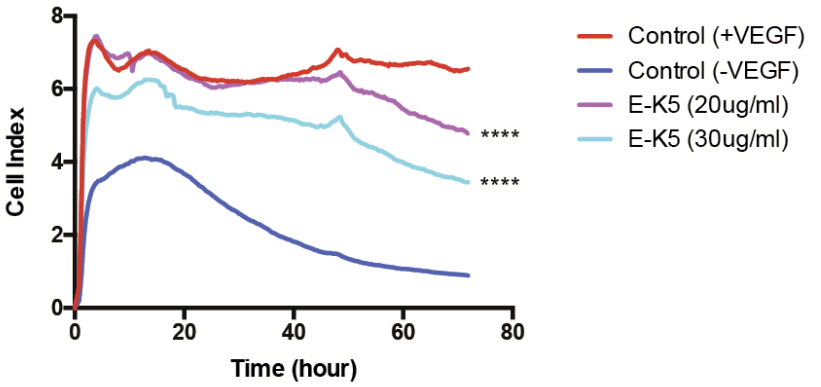


Figure 3. Effect of E-K5 on endothelial cell proliferation. A and B. Representative real-time tracings of HUVEC-I and HUVEC proliferation respectively.
E-K5 (20ug/ml and 30ug/ml) showed inhibition of VEGF-A (100ng/ml) induced EC proliferation. Within 20 hours, wells treated with E-K5 showed a concentration dependent inhibition of proliferation. Quantitation of the inhibitory effect of E-K5 on VEGF-A induced proliferation of HUVEC-I and HUVEC is based on two independent real time experiments. Statistical analyses were performed using GraphPad Prism® 6. Differences in mean values between the two groups were analyzed using the two-tailed Student's t-test.
*P ≤ 0.05 **P ≤ 0.01 ***P ≤ 0.001 ****P ≤ 0.0001 compared to control.

E-K5 inhibits VEGF-A induced endothelial cell migration.

To further study the biological activity of the fusion protein on endothelial cell activity, effect on VEGF-A induced migration was determined. First, scratch wound assays were performed. Endothelial cells were serum starved and then treated with 100ng/ml VEGF-A to induce cell migration. Sets of cultures were either treated with P125A endostatin, Kringle 5 or E-K5. Wells treated with K5 inhibited ~ 25% of VEGF-A induced EC migration; treatment with P125A alone showed ~ 35% inhibition whereas; E-K5 treatment inhibited ~ 50% of migrating cells (Figure 4A and B). These results suggest that, treatment with E-K5 had a better biological activity against migrating endothelial cells compared with P125A or K5 alone.

In a parallel series of experiments, we determined the concentration-dependent effect of E-K5 on EC migration. Serum starved HUVEC-I were stimulated with 100ng/ml VEGF-A. In the presence of even 10ug/ml E-K5 there was considerable inhibition of migration of cells in 24 hours. Twenty ug/ml E-K5 progressively decreased migration of EC (Figure 4C and D).

To further validate these findings, Cell migration was determined in a Boyden Chamber assay. Migration of HUVEC- I across an 8um pore size membrane towards VEGF-A gradient containing EBM-2 medium was determined. After

12 hours of incubation, the inner chamber was washed and the cells were fixed using 4% PFA. To accurately determine the number of cells attached to the lower face of the membrane, the upper face was gently swabbed and the membrane was stained with DAPI for imaging the number of cells that had migrated across the gradient. These studies showed that E-K5 inhibited the migration of HUVEC- I in a concentration dependent manner. Both 10ug/ml and 20ug/ml showed significant inhibition of cells that had migrated across the membrane compared to control wells (Figure 4 E and F). These results indicate that E-K5 inhibited VEGF-A induced endothelial cell migration as shown by both scratch wound and Boyden chamber assay.

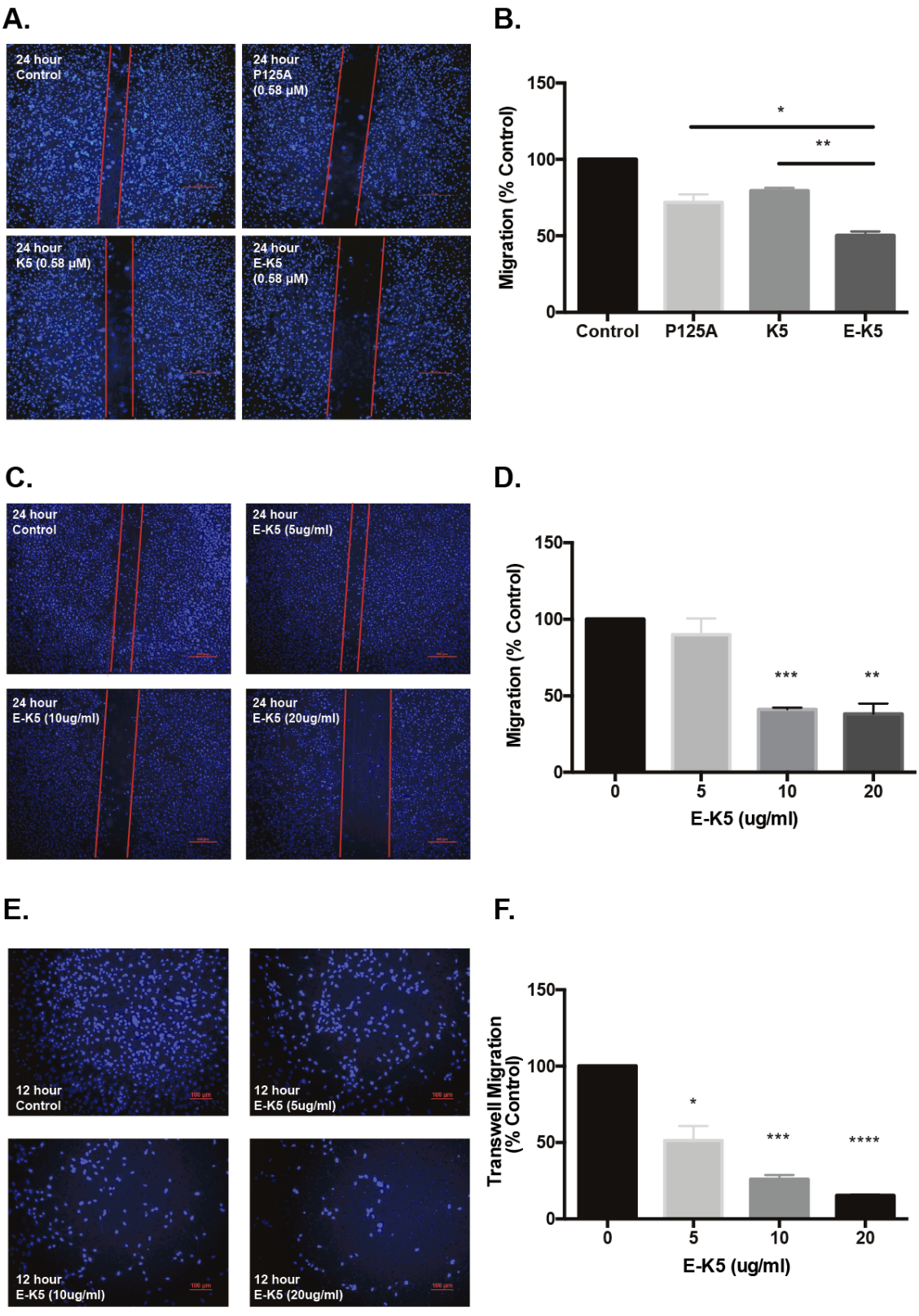

A.
24 hour Control
24 hour P125A (0.58 μM)
24 hour K5 (0.58 μM)
24 hour E-K5 (0.58 μM)
B.
Migration (% Control)
150
100
50
0
*
**
Control
P125A
K5
E-K5
C.
24 hour Control
24 hour E-K5 (5ug/ml)
24 hour E-K5 (10ug/ml)
24 hour E-K5 (20ug/ml)
D.
Migration (% Control)
150
100
50
0
***
**
0
5
10
20
E-K5 (ug/ml)
E.
12 hour Control
12 hour E-K5 (5ug/ml)
12 hour E-K5 (10ug/ml)
12 hour E-K5 (20ug/ml)
F.
Transwell Migration (% Control)
150
100
50
0
*
***
****
0
5
10
20
E-K5 (ug/ml)

Figure 4. Effect of E-K5 on HUVEC-I migration. A and B. Scratch wound assay of HUVEC-I treated with P125A, K5 and E-K5. By 24 hours, control cells stimulated with VEGF-A showed ~95% closure of the wound. Treatment with P125A and K5 showed a small inhibition of migration. Cells treated with E-K5 showed 50% inhibition of VEGF-A induced migration (n=2). **C and D.** Scratch wound assay of HUVEC-I with increasing concentrations of E-K5. A decrease in VEGF-A induced migration in both 10ug/ml and 20ug/ml E-K5 treated groups compared with control groups by 24 hours, which was confirmed as statistically significant when quantified using Image J software. All images were viewed under a fluorescence microscope at 4x magnification at 0 and 24 hours (n=3) **E and F.** VEGF-A induced EC migration was measured using a Boyden chamber assay. E-K5 treated cells showed inhibition of migration compared to control wells. All images were viewed under 16x magnification and 5 random fields per well were captured. Cells were counted automatically using Image J (n=2). Data are expressed as means ± SD. Statistical analyses were performed using GraphPad Prism® 6. Differences in mean values between the two groups were analyzed using the two-tailed Student's t-test. *P ≤ 0.05 **P ≤ 0.01 ***P ≤ 0.001 ****P ≤ 0.0001 compared to control.

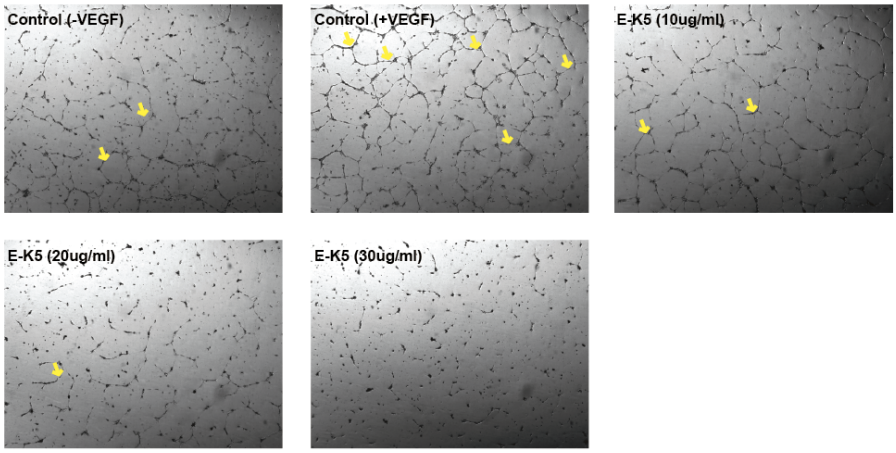
E-K5 inhibits VEGF-A induced tube formation and vessel sprouting in vitro.

Since E-K5 inhibited the proliferation and migration of HUVEC-I, we further studied its effect on endothelial cell morphogenesis. To assess its biological activity on endothelial tube formation, serum starved HUVEC-I were plated on a basement membrane (Geltrex) and stimulated with VEGF-A to form capillary-like tubes. Growth factor staved HUVEC-I with no stimulus (VEGF-A) showed reduced formation of tubes and had decreased nodes and junctions. In the presence of increasing concentrations of E-K5, there was significant inhibition of tube formation. Both 20ug/ml and 30ug/ml of E-K5 showed ~ 50 % inhibition of

VEGF-A induced tube formation (Figure 5A and B) compared to cells stimulated with VEGF-A alone.

Then we investigated the effect of E-K5 in a three- dimensional in vitro sprouting angiogenesis model, which recapitulates the important processes involved in angiogenesis namely budding, cell migration, cell proliferation, branching and anastomosis. Cytodex beads were coated with HUVEC-I and embedded in Geltrex and the formation of sprouts was observed over a period of 6 days. Beads were treated with E-K5 every other day. On day 6 we observed that in E-K5 treated fibrin gels, there was inhibition of more than 80% of number of sprouts and also a significant decrease in the length of the sprouts. Moreover, treatment with 20ug/ml E-K5 failed to produce sprouts with a length greater than bead diameter (Figure 5 C and D). In summary, these experiments demonstrated the ability of E-K5 to inhibit EC sprouting.

A.



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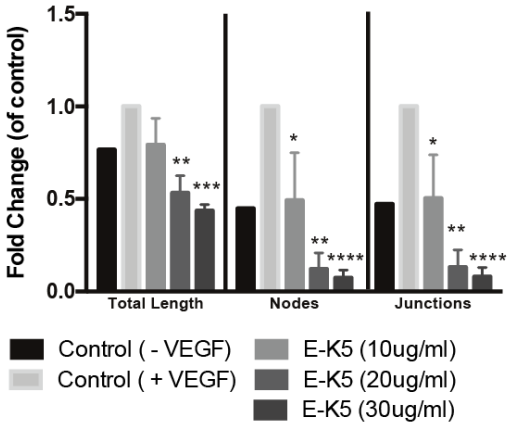
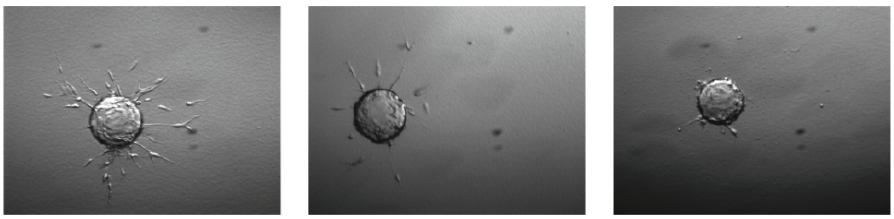


Figure 5. Effect of E-K5 on HUVEC-I morphogenesis- Tube formation.
A and B. Growth factor starved HUVEC- I were stimulated with VEGF-A (100ng/ml) and seeded on Geltrex coated plates. After 16 hours phase contrast images were taken to evaluate the effect of E-K5 on HUVEC-I tube formation. Control cells showed formation of capillary tube like structure with intracellular spaces. Treatment with E-K5 (30ug/ml) completely inhibited tube formation of HUVEC-I. Results shown represent values from three independent experiments. Data are expressed as means ± SD. Statistical analyses were performed using GraphPad Prism® 6. Differences in mean values between the two groups were analyzed using the two-tailed Student's t-test.
*P ≤ 0.05 **P ≤ 0.01 ***P ≤ 0.001 ****P ≤ 0.0001 compared to control.

C.



D.

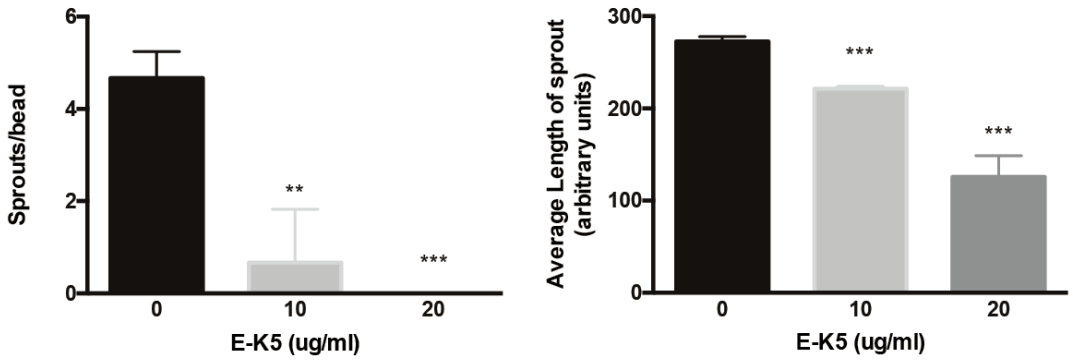
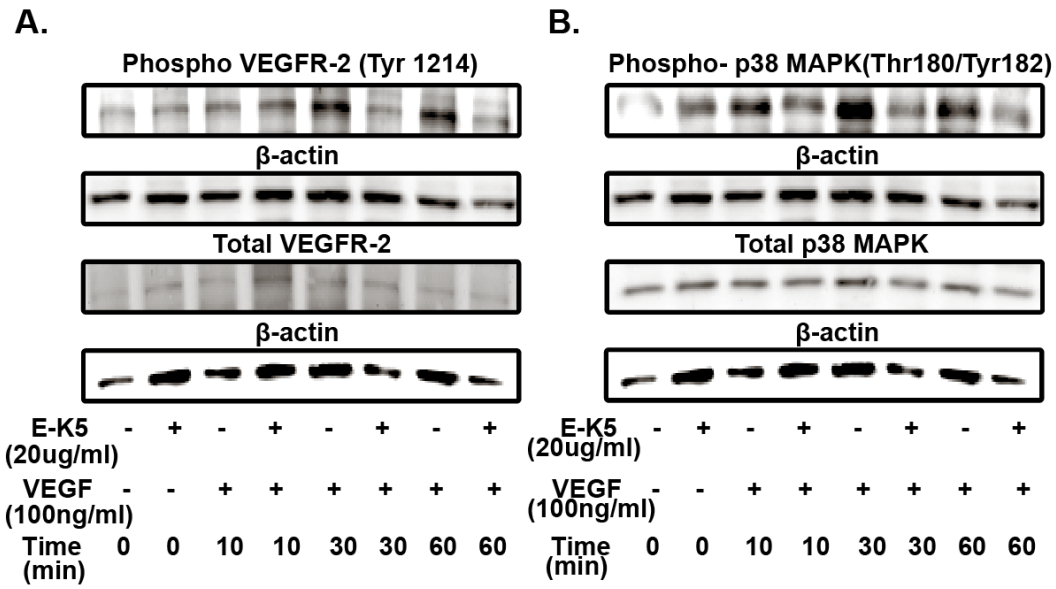


Figure 5. Effect of E-K5 on HUVEC-I morphogenesis- Sprouting Angiogenesis.
C and D. Three dimensional sprouting angiogenesis. Cytodex beads were coated with HUVEC-I and cultured for 6 days. Beads treated with E-K5 show decreased sprout length and number of sprouts. Sprouts were not counted if their diameter was not greater than bead diameter. Images were taken under 10x magnification and at least 5 random fields were captured. Experiment was repeated three independent times and representative data are shown. Data areexpressed as means ± SD. Statistical analyses were performed using GraphPad Prism® 6. Differences in mean values between the two groups were analyzed using the two-tailed Student's t-test.
*P ≤ 0.05 **P ≤ 0.01 ***P ≤ 0.001 ****P ≤ 0.0001 compared to control.

E-K5 treatment decreases VEGFR-2 phosphorylation and downstream signaling events.

In order to understand the mechanism by which E-K5 inhibits VEGF-A proliferation and migration, we investigated the effect on intracellular signaling pathways induced by VEGF-A- VEGFR-2 system. Major phosphorylation sites on VEGFR-2 are namely Y1175 and Y1214. Phosphorylation of tyrosine 1214 is required for actin polymerization and cell migration and hence we studied the effect of E-K5 on VEGF-A induced phosphorylation of VEGFR-2. Growth factor starved and E-K5 treated HUVEC-I were stimulated with 100ng/ml VEGF-A and lysates were collected at the specified time intervals. Figure 6 A and C shows that E-K5 significantly inhibited VEGF-A stimulated phosphorylation of Tyr- 1214 after 30 and 60 minutes. Next, we looked at events downstream of Tyr-1214 after E-K5 treatment. Figure 6 B and D show that, when HUVEC-1 were treated with E-K5, phosphorylation of p 38 MAPK (Thr 180/Tyr 182) was also decreased. These results suggest that, E-K5 inhibits cell migration by blocking the phosphorylation of Tyr-1214 and the activation of its downstream target p38 MAPK. Among multiple signaling molecules contributing to cell migration, phosphorylation of FAK is important. Interestingly, E-K5 treatment inhibited phosphorylation of FAK at Tyr-861 but not at Tyr-397 (Figure 7 C, D and 9A) suggesting that it may hinder exclusively Src mediated phosphorylation of FAK (46). Next we looked at the survival pathways induced by activation

VEGFR-2. Activation of AKT and p44/42 MAPK occurs upon VEGF-A stimulation (Figure 7 A and E), which was decreased by E-K5 treatment. Phosphorylation of AKT at Ser 473 decreased significantly at 10, 30 and 60 min after VEGF-A stimulation, hindering the survival of endothelial cell upon E-K5 treatment.



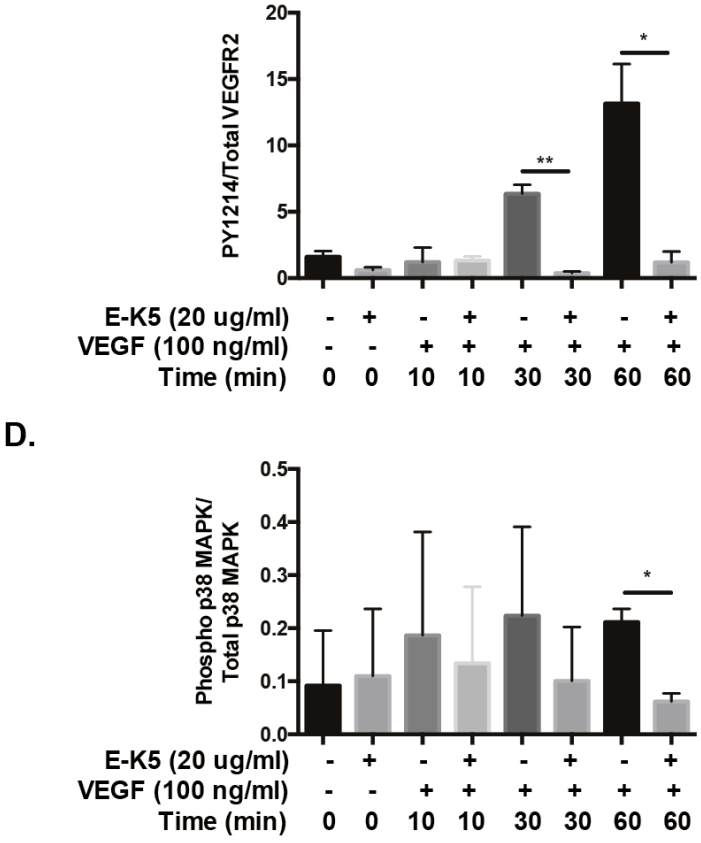


Figure 6. Effect of E-K5 on VEGF-A induced signaling. Whole cell lysates of HUVEC-I were growth factor starved and treated with E-K5 overnight. Cells were treated with VEGF-A and were evaluated for phosphorylation of VEGFR-2 **(A and C)** by western blotting. **B and D.** Lysates were also analyzed for phosphorylation of p-38 MAPK. β-Actin was used to normalize the values of phospho and total protein levels. Data represents values from two independent experiments. Statistical analyses were performed using GraphPad Prism® 6. Differences in mean values between the two groups were analyzed using the two-tailed Student's t-test. *P ≤ 0.05 **P ≤ 0.01 ***P ≤ 0.001 ****P ≤ 0.0001

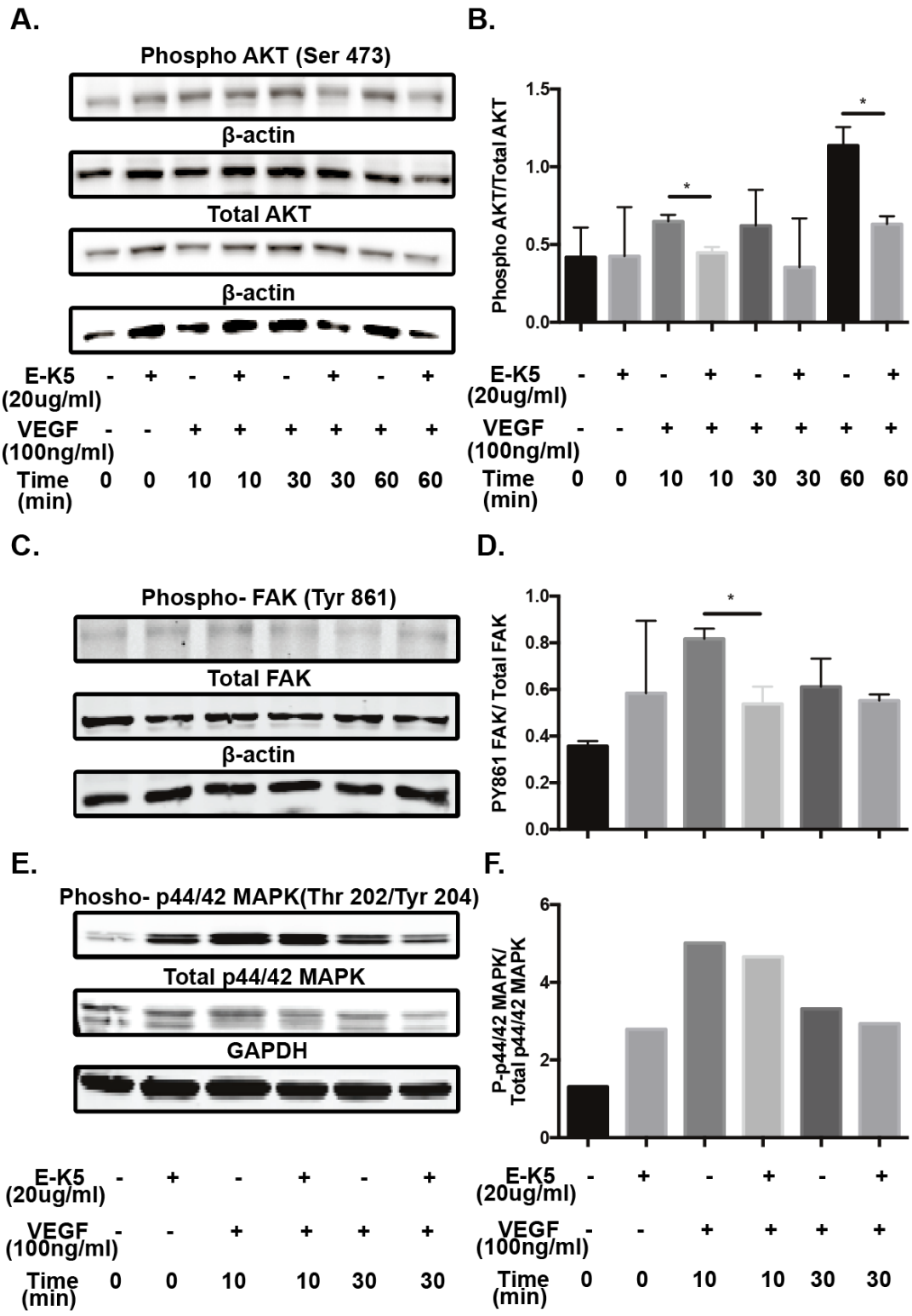


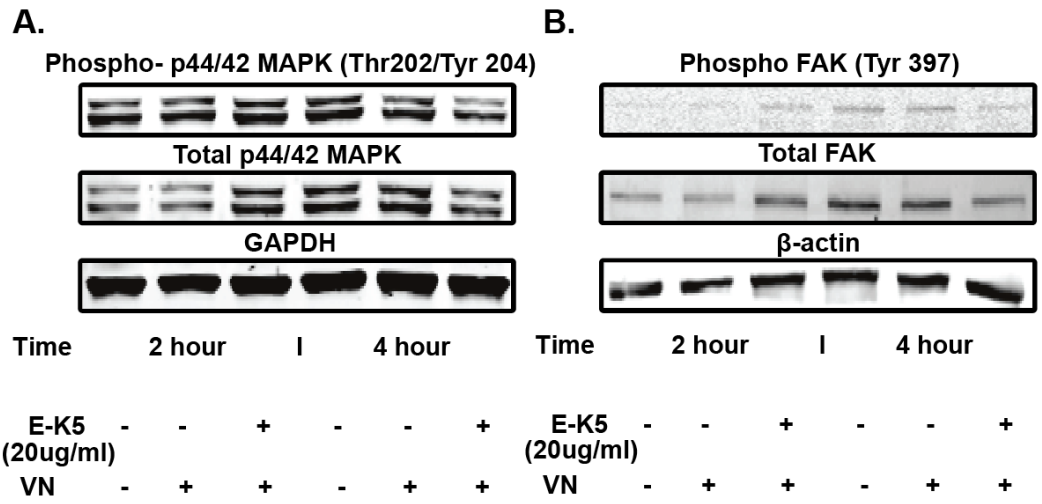
Figure 7. Effect of E-K5 on VEGF-A induced signaling. **A and B.** Phospho-AKT (Ser 473)

levels. Cells pretreated with E-K5 showed decrease in phosphorylation after 10, 30 and 60 min of VEGF-A treatment. Data represents values from two independent experiments. **C and D.** Phospho-FAK (Tyr 861) levels (n=2) **E and F.** P-p44/42 MAPK levels (n=1). Statistical analyses were performed using GraphPad Prism® 6. Differences in mean values between the two groups were analyzed using the two-tailed Student's t-test. *P ≤ 0.05 **P ≤ 0.01 ***P ≤ 0.001 ****P ≤ 0.0001.

E-K5 treatment decreases vitronectin-induced integrin signaling.

Since previous experiments proved that E-K5 treatment inhibits cell attachment by the involvement of integrin αvβ3 to a great extent, we further investigated if integrin signaling is inhibited. HUVEC-I cells were seeded on non-gelatin coated dishes, to eliminate any external stimulus. Once sub-confluent, they were growth factor starved and after 16 hours, re-plated on vitronectin-coated dishes with or without E-K5 treatment. Vitronectin is the main ligand for integrin αvβ3 on endothelial cells and we hypothesized that E-K5 treatment would decrease the ligand-induced activation of αvβ3. According to the real time cell attachment studies (Figure 2 B), we looked at an early time point (2 hour) and later time point (4 hour) to accurately assess the signaling events. Phosphorylation of p44/42 MAPK occurs due to the following signaling events upstream a. Ras activation due to growth factor receptor stimulation (VEGFR-2), b. FAK phosphorylation due to activation of integrin outside-in signaling, c. Activation of SFKs due to integrin signaling, which feeds growth factor signaling and results in activation of

p44/42 MAPK (47). Thus, p44/42 MAPK activation serves as a nodal point between VEGR-2 and αvβ3. Consequently, we looked at the phosphorylation state of p44/42 MAPK on stimulation of αvβ3. After 2 hours, we observed a decrease in the phosphorylation of p44/42 MAPK and after 4 hours a further decrease in the phosphorylation state on E-K5 treated cells (Figure 8 A and C). Next we looked at Focal adhesion kinase which is phosphorylated after external integrin signaling. Even though there are 6 phosphorylation sites on FAK - Tyr 397, 407, 576, 577, 861 and 925, upon integrin engagement by ligand, FAK is recruited and autophosphorylation occurs at Tyr 397. Interestingly, in contrast to VEGFR-2 signaling results, phosphorylation at Tyr 397 was decreased significantly after 4 hours of attachment (Figure 8 B and D) but E-K5 did not have any significant effect on the phosphorylation state at the Src family kinase (SFK) dependent activation site Tyr 861 (Figure 9B).



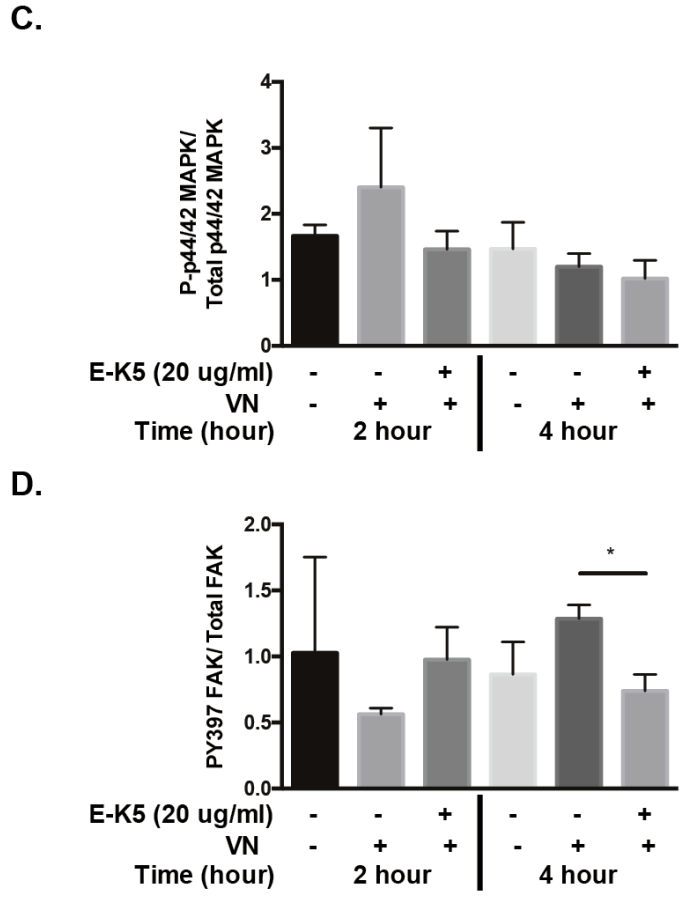


Figure 8. Vitronectin induced integrin signaling.
HUVEC-I were growth factor starved ovenight and plated on VN coated dishes. Lysates were collected 2 and 4 hours after cell attachment.
A and C. When cells were treated with VN they showed an activation of signaling molecule P- p44/42 MAPK by 2 hours and E-K5 treatment inhibited this activation .
B and D. Interestingly, FAK activation occured only after 4 hours of VN stimulus and E-K5 treatment showed a significant decrease in phophorylation of FAK (Tyr 397). Data represents values from two independent experiments. Statistical analyses were performed using GraphPad Prism® 6. Differences in mean values between the two groups were analyzed using the two-tailed Student's t-test.
*P ≤ 0.05 **P ≤ 0.01
***P ≤ 0.001 ****P ≤ 0.0001.

A.

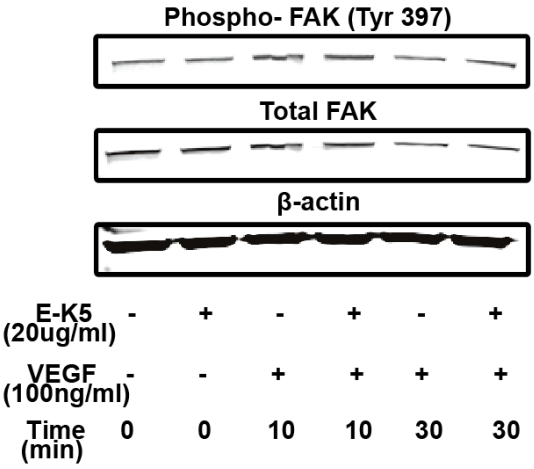


Figure 9 A. Effect of E-K5 on VEGF-A induced phosporylation of FAK at Tyr 397. Cells treated with E-K5 did not inhibit the phosphorylation of FAK at Tyrosine 397.

B.

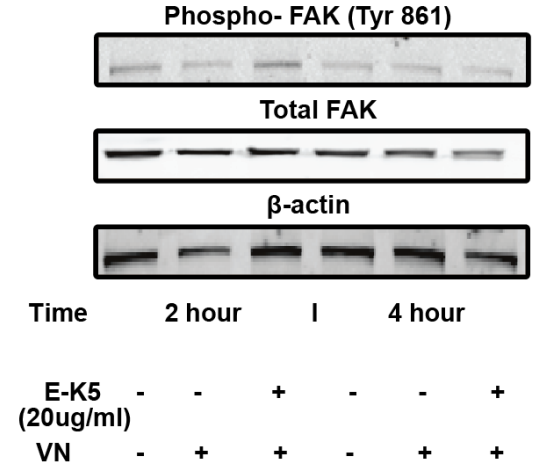


Figure 9B. Effect of E-K5 on Vitronectin induced phosporylation of FAK at Tyr 861. E-K5 treatment did not have any significant effect at the SFK dependent activation site Tyr 861 at both 2 and 4 hours.

E-K5 inhibits tumor-induced angiogenesis.

After establishing the mechanism of inhibition of E-K5, we then determined whether E-K5 can inhibit tumor cell-induced angiogenesis *in vivo*. Using Lewis Lung Carcinoma (LLC) Matrigel plugs as our model we investigated if systemic delivery of E-K5 decreases *in vivo* angiogenesis. LLC containing Matrigel plugs were injected s.c. into C57/bl6 mice. Mice were treated with either saline or E-K5 at indicated doses. Matrigel plugs were resected from mice after 10 days and snap frozen. Frozen sections were prepared and stained with anti-CD 31 and α-SMA antibody conjugated to fluorescent dyes. CD31 positive vessels were quantified by image analyses as described in the *Materials and Methods*. E-K5 treatment significantly decreased both vessel length and nodes (Figure 10 A and B) and treated mice had a ~ 2-fold increase in pericyte coverage (Figure 10 C and D). The results form this study show that, E-K5 effectively inhibited tumor angiogenesis and inhibited premature vessels more selectively.

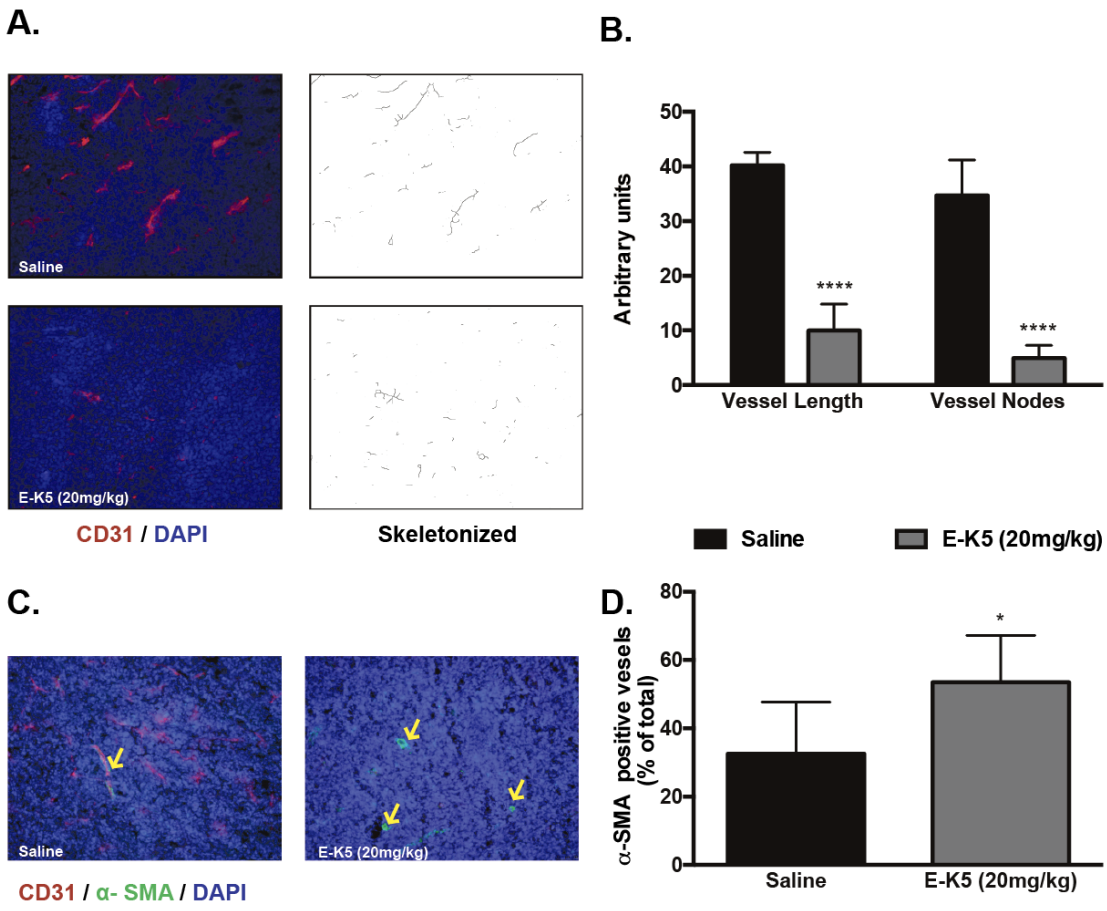


Figure 10. Anti-angiogenic activity of E-K5 fusion protein. Lewis Lung Carcinoma matrigel plugs were implanted s.c in C7BL/6 mice. Mice were treaed with saline or fusion protein E-K5. Frozen sections were stained with anti-mouse CD 31 Ab (red) for blood vessels, anti- α-SMA Ab to show mature vessels and DAPI to show nuclei. Each group had atleast 8 mice. **A and B**.Vessel density was determined by morphometric analysis.
C and D. CD 31 positive vessels co stained with α- SMA were quantified for mature vessel percentage. Statistical analyses were performed using GraphPad Prism® 6.
Differences in mean values between the two groups were analyzed using the two-tailed Student's t-test.
*P ≤ 0.05 **P ≤ 0.01 ***P ≤ 0.001 ****P ≤ 0.0001 compared to control.

Discussion

Anti-angiogenic therapy was originally proposed by Dr. Judah Folkman to improve cancer treatment strategies such as adjuvant chemotherapy (48). Despite significant efforts to develop new and improved angiogenesis inhibitors, patients showed only a partial clinical response (44). Therefore, there is a need for improving anti-angiogenic treatments. Efficacy of anti-angiogenic treatment is limited by the bioavailability and potency of angiogenesis inhibitors. To overcome these limitations, endostatin was genetically modified to improve therapeutic efficacy. In our studies, we used a fusion construct of two anti-angiogenic proteins/peptides to generate a chimeric protein. Fusion protein was found to inhibit angiogenesis very efficiently. In a recently published study, anti-angiogenic proteins were targeted to tumor vasculature by combining, a novel recombinant CTT peptide-endostatin mimic with kringle 5. This construct was found to inhibit MMPs and endothelial cells. (49).

VEGF-A has been recognized as an important growth factor that protects tumor vessels from undergoing apoptosis. Additionally, the crosstalk between VEGFR-2 system and integrin receptors is well recognized to play an important role in angiogenesis (22). Previous studies have shown that endostatin mainly interacts with α5β1 and another collagen derived anti-angiogenic protein, tumstatin engages αvβ3 and inhibit angiogenesis. In the present study, we show that fusing mutant endostatin with kringle 5 of plasminogen enhances its biological

activity. Fusion protein inhibited endothelial cell proliferation and migration by targeting multiple signaling pathways. We evaluated whether E-K5 inhibited both integrin signaling and VEGFR-2 signaling. Our studies show a dramatic decrease in endothelial cell attachment to fibronectin and vitronectin when compared to mutant endostatin alone. These studies showed the involvement of the αv and α5 family of integrin heterodimers. Present studies further show that E-K5 binds to not only α5β1 but also to αvβ3 and inhibits endothelial cell attachment. E-K5 was found to inhibit activation of specific downstream targets of αvβ3 signaling, Tyr 397 FAK and P-p44/42 MAPK. Interestingly, previous studies have shown that FAK is central in regulation of αvβ3 and VEGFR-2 signaling, though how this crosstalk occurs has not been elucidated. A primary event after integrin engagement to ECM components is phosphorylation of FAK at Tyr 397. Similarly in RTKs, VEGF-A via Src kinase causes site-specific phosphorylation of FAK at Tyr 861. There is some evidence to show that, Src coordinates the VEGF induced activation of FAK at Tyr 861 leading to a signaling complex formation between FAK/ α5β1 (50). Our results suggest that E-K5 specifically inhibits FAK phosphorylation at Tyr 861 on VEGF–VEGFR-2 activation and FAK 397 on αvβ3 activation. These results suggest that E-K5 may interfere with the recruitment of αvβ3 to FAK due to decreased activation at Ty861 resulting in inhibition of phosphorylation of FAK 397 due to reduced αvβ3 engagement. Further studies will evaluate VEGF induced FAK/ αvβ3 complex formation and the role of

Src kinase in the formation of the inhibitory complex. Our studies also show that E-K5, inhibits the phosphorylation of VEGFR-2 at Tyr 1214, a site important for cell migration.

To further examine the in vivo efficacy of the fusion protein, we studied if systemic E-K5 treatment inhibited tumor-induced angiogenesis. Our data shows that, E-K5 treatment reduced vessel density and normalized tumor vasculature. Increased pericyte coating in residual blood vessels of the Matrigel plugs indirectly suggest vessel normalization. Vessel normalization is considered to improve blood flow, which is necessary for drug delivery and therapeutic efficacy. Indeed, Bevicizumab treatment and the use of anti-VEGFR-2 antibodies normalized tumor vasculature and increased delivery of chemotherapeutic drugs such as doxorubicin. Since E-K5 has shown to inhibit endothelial cell attachment in vitro in numerous experimental designs, future studies will look into the in vivo efficacy of E-K5 on peritoneal seeding of ovarian tumor cells and metastasis. It will be interesting to determine whether E-K5 treatment could improve chemo delivery and efficacy. It is also important to study the differential effects of P125A, K5, E-K5 on the gene expression profiles of HUVEC. Comparison of mRNA and miRNA expression profile following E-K5 treatment can be used to identify additional targets for drug development. Furthermore, these investigations will identify compensatory mechanisms that are upregulated during anti-angiogenic treatment with E-K5.

Interestingly, the addition of Kringle 5 to mutant endostatin did not sterically hinder the biological activity of P125A. Our studies have shown that fusion protein, E-K5 has improved biological activity against endothelial cells when compared to P125A or K5 alone. This raises the possibility that fusion protein, E-K5 can also associate with GRP78 and VDAC1, which are the targets for K5. Independent binding of P125A-endostatin to integrins and K5 binding to GRP78/VDAC1 could generate distinct signals and result in improved efficacy of the fusion protein. This possibility needs validation using direct binding studies using molecularly tagged proteins. It would be interesting to investigate whether E-K5 treatment could also cause programmed cell death.

In conclusion, our results show that genetic fusion of endostatin to Kringle 5, generated a potent anti-angiogenic molecule. The fusion protein inhibited endothelial cells effectively by targeting both VEGF- VEGFR-2 system and αvβ3 integrin-mediated signaling pathways. Fusion protein was effective in inhibiting tumor angiogenesis in vivo. Future studies will evaluate the effect of E-K5 on tumor growth and metastasis in preclinical models.

Materials and Methods

Cell Lines and Reagents

Human Umbilical Vein Endothelial Cells immortalized with SV40 antigen (HUVEC-I), kindly were provided by Dr. A. Kelekar. Primary Human Umbilical Vein Endothelial Cells (HUVEC), between passages 2-4, were obtained from Neuromics (Edina, MN). Endothelial cells were grown in tissue culture flasks coated with 0.2% gelatin and maintained in Endothelial cell Growth Media EGM-2™ (Lonza). Lewis Lung Carcinoma (LLC) cells were provided by Dr. S. Roy and cultured in RPMI 1640 (Life Technologies) supplemented with 10% FBS. VEGF-A was cloned and expressed in yeast (51). Fibronectin (FN), Vitronectin (VN), Laminin (LN) (Sigma) and Collagen Type 1 (BD Biosciences) were used in cell attachment studies. Cytodex 3 microcarriers (GE healthcare Lifesciences) were used in fibrin bead assay.

Purification of Recombinant Proteins

Pichia clones were cultured in baffled flasks and protein expression was induced by methanol induction every 24 hours upto 3 days. P125A-endostatin-Kringle 5 (E-K5) fusion protein was purified as described previously using HiTrap Heparin HP Columns (GE Healthcare) (52). Bound protein was eluted with a continuous gradient of 0-1 M NaCl in 10mM Tris-HCl, pH 7.6 0.5mM phenylmethylsulfonyl

fluoride (PMSF) by Fast Performance Liquid Chromatography. E-K5 was eluted at ≈ 0.5 M NaCl. Desalted fractions were resolved in SDS-PAGE gels (12%) and stained with Comassie blue.

Effect of E-K5 on Cell Attachment

Cell attachment studies were carried out using the Real-Time Cell Analyzer Dual Plate (RTCA DP) Instrument (ACEA Biosciences) according to the manufacture's suggested protocol with modifications. E-plate 16 (ACEA Biosciences) were coated with Fibronectin (FN) (10ug/ml) or Vitronectin (VN) (5ug/ml) and incubated overnight at 4^{o} C and blocked with 2% BSA in PBS for 30 minutes. Uncoated wells were blocked with BSA and used as negative control. HUVEC-I were serum starved for 12 hours in Endothelial Cell Basal Medium-2 (EBM-2). Cells (15,000) were treated with or without E-K5 (20ug/ml) in EBM-2 medium and seeded on E-plates 16. Impedance measurement was recorded every 15 minutes for 24 hours. In a modified cell attachment assay, HUVEC-I were suspended in EBM-2 and incubated in the presence of antibody to αvβ3 or IgG control at 37^{o} C for 30 minutes. They were further incubated with or without E-K5 (20ug/ml) at 37^{o} C for 30 minutes. Cells were seeded on VN coated E-plate 16 and impedance measurement was recorded.

Direct Binding of E-K5 to HUVEC-I

96 well black with clear bottom microtiter wells were coated overnight at 4^{o} C with 20ug/ml of E-K5. The wells were blocked with 2% BSA in PBS for 30 minutes. Trypsinized HUVEC-I were labeled using EBM-2 medium containing 20 uM cell tracker blue dye and incubated at 37^{o} C for 30 min. The dye containing media was removed and cells were washed with HBSS. HUVEC-I were re-suspended in EBM-2 medium with or without anti-integrin antibodies and incubated at 4^{o} C for 30 min. 10,000 cells were added to the wells and the plates were incubated at 37^{o} C for 2 hours. The wells were gently washed twice with HBSS and fluorescence intensity was measured using Tecan Infinite M1000 Microplate Reader. Background emission from uncoated wells was subtracted from the obtained values.

Effect of E-K5 on VEGF-A induced cell proliferation

HUVECS-I and HUVECS (15,000) were serum starved overnight in EBM-2 with 2% serum. Cells were then treated with or without E-K5 (20ug/ml and 30ug/ml) in EBM-2 medium with 2% serum and 100ng/ml VEGF-A. Cells were added to gelatin coated E-plate 16 and impedance measurement was recorded using the RTCA DP Instrument.

Effect of E-K5 on VEGF-A induced migration (Scratch Wound Assay)

Cells (50,000) were seeded onto gelatin coated, 24 well plates. HUVECS-I were serum starved as described previously. Cells were treated with P125A, K5 or E-K5 (10ug/ml and 20ug/ml) and 100 ng/ml VEGF-A in EBM-2 medium with 2% serum and pipette tip was used to make scratch wounds. Images were taken at 0 hours and 24 hours using Nikon AZ100M Macro Fluorescence Microscope. Absolute migration in 24 hours was quantified using Image J 1.6.0_65. Each sample was assayed in triplicate and the data represent values from 3 independent experiments.

Effect of E-K5 on VEGF-A induced migration (Boyden Chamber assay)

Eight um pore size transwell inserts were used for this assay (BD Falcon). HUVEC-I (10,000) were suspended in EBM-2 with 2% serum and different concentrations of E-K5 protein and added to the upper chamber of the assembly. EBM-2 with 2% serum and 100ng/ml VEGF-A was added to the lower chamber. Wells without VEGF-A were used as negative control. Cells were allowed to migrate for 12 hours at 37^{o} C and 5% CO_2. Cells attached to the lower side of the membrane were fixed with 4% Paraformaldehye (PFA). The membrane was washed with PBS and stained with DAPI. Images were taken at 12x magnification using Nikon AZ100M Macro Fluorescence Microscope. At least 5 random fields

per well were imaged and each sample was assayed in triplicate and the experiment was repeated twice.

Effect of E-K5 on VEGF-A induced Tube Formation assay

HUVEC-I were serum starved for 8 hours as described previously. Twenty-four well plates were coated with 250 ul of Geltrex® Low-growth factor basement membrane matrix (Life Technologies). HUVECS-I (50,000) were treated with E-K5 (10ug/ml, 20ug/ml and 30ug/ml) in EBM-2 with 2% serum and 100ng/ml VEGF-A and incubated for 16 hours. Four random fields per well were imaged using 4x magnification of the Nikon AZ100M Macro Fluorescence Microscope. Tube length, nodes and junction was quantified using the Image J Angiogenesis (1.0.c) analyzer plugin. Data represent values from 2 independent experiments.

Effect of E-K5 on Fibrin Bead assay.

HUVEC and HUVEC-I were maintained in EGM-2 medium and seeded on gelatin coated cytodex-3 micro carriers as described previously for a period of 4 hours (53). Endothelial cell sprouting was induced in the presence of EGM- 2 media with or without E-K5 (10ug/ml and 20ug/ml) every other day. Bead cultures were imaged on day 6 using Leica DM IL microscope. Sprout number was quantified by scoring sprouts with lengths greater than bead diameter using Image J 1.6.0_65 (National Institutes of Health). Sprout length was also computed in arbitrary

units. Data represent values from 3 independent experiments.

VEGF-A induced signaling

HUVEC-I were plated on gelatin-coated dishes and maintained in EGM-2 medium. After they reached 80 % confluency, they were treated overnight with or without E-K5 (20ug/ml) in EBM-2 with 2 % serum. After 16 hours of treatment, they were stimulated with 100ng/ml VEGF-A and lysates were collected after 0, 10, 30 and 60 minutes using RIPA buffer supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein concentration was measured using BCA Protein Assay Kit (Pierce) and 25ug protein was loaded. Samples were resolved in 7.5% and 12% SDS-PAGE gels and transferred to a nitrocellulose membrane. Blots were blocked with 5% non-fat milk or 5% BSA in TBST (phospho antibodies) or odyssey blocking buffer for an hour. Immunoblots were detected using Clarity western ECL Kit (Bio Rad) or Odyssey Imager. Densitometric analyses were done using ImageJ software.

Vitronectin-induced signaling

HUVEC-1 were grown on non-gelatin coated dishes and maintained in complete endothelial growth media. They were starved overnight in EBM-2 medium with 2 % media. After 16 hours, cells were trypsinized and suspended in EBM-2 with 2

% serum in presence or absence of E-K5 (20ug/ml) and re-plated on Vitronectin coated dishes. Lysates were collected after 2 hours and 4 hours using RIPA buffer.

Effect of E-K5 on Tumor angiogenesis

Female C57BL/6 (6-8 weeks) were used for the Tumor induced angiogenesis study. LLC cell suspension in PBS (1 million cells/plug) was mixed with equal volume of Matrigel and injected into the dorsal flanks subcutaneously (bilateral plugs). Mice were randomized into two groups on day four. One group of mice was injected with E-K5 (20mg/kg/day) i.p. for 8 days. On day 9 mice were euthanized and plugs were weighed, harvested and imaged. Frozen sections (15um) of Matrigel plugs were fixed in cold acetone for 10 minutes and stained with PE- conjugated anti- mouse CD31 antibody (1:100), FITC-conjugated α-SMA and counterstained with DAPI. Slides were analyzed for vessel density by imaging (7-10) random frames per section using 20x magnification of Leica DM5500 B microscope. CD 31-positive vessel area and branch points per vessel were quantified as described previously (54).

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