Antiangiogenic Activity of a Domain Deletion Mutant of Tissue Plasminogen Activator Containing Kringle 2

Veronica A. Carroll, Leonid L. Nikitenko, Roy Bicknell, Adrian L. Harris

Objective—The thrombolytic therapy drug, Reteplase, is a domain deletion mutant of tissue plasminogen activator (tPA), comprising the kringle 2 and protease (K2P) domains. Some kringle domains of hemostatic proteins are antiangiogenic and promote apoptosis. The objective of this study was to investigate whether K2P is an angiogenesis inhibitor because of the presence of kringle 2.

Methods and Results—K2P inhibited basic fibroblast growth factor-induced human endothelial cell proliferation and migration. Inhibition was not dependent on the protease activity of K2P because similar results were obtained with catalytically inactivated K2P. Purification of the kringle 2 domain derived from elastase cleavage of K2P at the Arg275–Ile276 bond revealed that inhibition was mediated by this domain. In addition, K2P inhibited angiogenesis in vivo and increased endothelial cell apoptosis.

Conclusions—Wound healing and angiogenesis are severely compromised by K2P. These data provide new mechanistic insights into the bleeding complications observed in some patients while undergoing thrombolytic therapy with this drug. In addition, we identify the kringle 2 domain of tPA as a novel target for antiangiogenic therapy. (Arterioscler Thromb Vasc Biol. 2005;25:736-741.)

Key Words: antiangiogenesis ■ reteplase ■ kringle 2 ■ endothelial cells ■ wound healing

Tissue plasminogen activator (tPA) is a serine protease that activates fibrinolysis through the conversion of plasminogen to plasmin. Plasmin, in turn, degrades fibrin in the vasculature during clot dissolution and wound healing.1

Plasminogen to plasmin. Plasmin, in turn, degrades fibrin in widespread use for treating thrombosis during acute myocardial infarction has led to the development of tPA derivatives with improved pharmacokinetic properties.5 One of these is tPA is expressed in a variety of cell types including endothelial cells and tumor cells.3,4 It is a multidomain protein that consists of a finger domain, an epidermal growth factor-like domain, 2 kringle modules, and a protease domain. Its widespread use for treating thrombosis during acute myocardial infarction has led to the development of tPA derivatives with improved pharmacokinetic properties.6 One of these is the domain deletion mutant comprising just the kringle 2 and protease domains (K2P) of tPA.

Kringles are triple-loop structures of 80 amino acids.7 Recent studies have shown that isolated kringle domains of proteins involved in the hemostatic system are antiangiogenic. These include plasminogen, which has 5 kringle domains,8 hepatocyte growth factor, which has 4,9 prothrombin, which has 2 kringles,10 and urokinase plasminogen activator (uPA), which has just 1.11 Angiotatin, consisting of kringle 1 to 2 domains of tPA on angiogenesis were not investigated in this study or the mechanism of action. In this report, we describe that the fibrinolytic drug, K2P, inhibits endothelial cell growth, migration, and in vivo angiogenesis. We show that inhibition is dependent on the kringle 2 domain and that K2P induces endothelial cell apoptosis in vivo.

Materials and Methods
Reteplase (domain deletion mutant of tPA comprising the kringle 2 and protease modules, otherwise known as Rapilysin or BM 06.022, but hereafter referred to as K2P) was purchased from Roche.
Purification of tPA Kringle 2 Domain

K2P was incubated with porcine pancreatic elastase in a molar ratio of K2P:elastase of 3.5:1 overnight at room temperature with gentle shaking. The digest was extensively dialyzed against column binding buffer (50 mmol/L tris-HCl, 50 mmol/L NaCl, pH 7.7) before addition of 1 mmol/L final concentration DTT and incubated for 3 hours at 37°C. Thereafter, the digest was applied to a lysis-Sepharose column previously equilibrated with binding buffer, washed, and the adsorbed kringle 2 domain was eluted in binding buffer containing 0.2 mmol/L e-amino-n-caproic acid. Eluted kringle 2-containing fractions were pooled and dialyzed with binding buffer to remove e-amino-n-caproic acid. To confirm purity, eluted kringle 2 was analyzed by SDS-PAGE. N-terminal sequence analysis, Western blot with polyclonal anti-tPA IgG #387 (American Diagnostica), and monoclonal anti-kringle 2 antibody TVPA (Technoclone Inc) at 1:1000 and 1:100 dilutions, respectively. Correct conformity of kringle 2 was confirmed by nuclear magnetic resonance. Possible contaminating endotoxin was removed from K2 preparations with PROSEP-RemTox (Millipore) according to manufacturer’s instructions.

Inactivation of K2P

Twenty-five mU K2P was catalytically inactivated with 2 rounds of either 2 mmol/L PMSF or 100 µmol/L PPACK, each for 1 hour at 37°C in inactivation buffer (50 mmol/L tris-HCl, pH 7.4, 100 mmol/L NaCl, 100 mmol/L l-arginine, 5 mmol/L EDTA, and 0.01% Tween 80), followed by extensive dialysis with the same buffer to remove excess inhibitor. To confirm inhibition of protease activity, 10 µL K2P were incubated with 50 µL Spectrozyme tPA chromogenic substrate (American Diagnostica) and 40 µL assay buffer (25 mmol/L tris-HCl, 200 mmol/L NaCl, pH 8.4) for 10 minutes at 37°C. Absorbance was measured at 405 nm.

In Vitro Cell Proliferation Assays

Cells (3×10^4) were seeded in 96-well plates and allowed to attach overnight. Cells were left in minimal growth medium (M199 with 2% FBS) for 24 hours. Thereafter, reagents were added to wells and incubated for 4 days. Cell number was quantified colorimetrically by the addition of 20 µL L Spectrozyme tPA (Promega) and measured at absorbance 492 nm.

Endothelial Cell Migration Assay

Endothelial cell migration was analyzed in a scratch wound assay as previously described with some modifications.21 Endothelial cells (3×10^3) were seeded in 96-well plates and allowed to attach overnight. Media was aspirated the next day and cells were scratched with a P200 pipette tip along the diameter of the dish. Cells were washed twice with PBS before addition of fractions of tPA in M199 and low serum (5% FBS) and incubated at 37°C and 5% CO2. Wound diameters were photographed at t=0, t=24 hours, and t=48 hours. Wound closure was determined by measuring the diameter of wounds at 3 equivalent points along the length of wounds and was expressed as a percentage of wound diameter at the start of the experiment.

Mouse Sponge Angiogenesis Assay

C57 black mice were implanted with a sterile polymer sponge (15×5×5 mm) under the dorsal skin as previously described.22 PBS, bFGF, or test reagents were injected daily through the skin into the sponge in a volume of 100 µL for 20 days. Sponges were dissected on day 21 and fixed in 3.7% formaldehyde, embedded in paraffin, cut in sections of 4 µm, and stained with hematoxylin and eosin. Morphometric analysis of fibrovascular growth (microvessels and fibroblasts) into sponges was performed as previously described with some modifications.23 A grid graticule of 1-mm² divisions with a total field area of 100 mm² was used to determine the volume fraction of fibrovascular growth contained in sponges.

Immunohistochemistry

Dewaxed and rehydrated paraffin sections were processed for immunohistochemistry as previously described with some modifications.22 Sections were microwaved in antigen retrieval buffer (0.01 mol/L sodium citrate, pH 6) for 15 minutes before incubation with rat-anti-mouse CD34 IgG (Ab8158; Abcam) or anti–Ki-67 (Vector Labs). Sections were subsequently incubated with biotinylated anti-rat IgG (DAKO) and horseradish peroxidase-conjugated avidin–biotin complex (DAKO) before staining with DAB (Sigma) and counterstaining with 50% Mayer hematoxylin. The total number of vessels in CD34-stained sponges were counted at ×20 magnification in 4 representative fields per sponge and data are given as mean±SD.

Apoptosis Assay

Apoptosis was determined on dewaxed and rehydrated sections with an in situ cell death detection kit (Roche) by means of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining according to manufacturer’s instructions. Briefly, sections were microwaved with 0.1 mol/L sodium citrate, pH 6, for 5 minutes before incubation with 0.1 mol/L tris-HCl, pH 7.5, containing 3% bovine serum albumin and 20% normal bovine serum for 30 minutes at room temperature. Slides were rinsed in PBS and incubated with 50 µL TUNEL reaction mixture for 1 hour at 37°C. Slides were rinsed with PBS, incubated with 50 µL converter POD for 30 minutes at 37°C, rinsed again, and processed with metal-enhanced DAB substrate (Sigma). Apoptotic cells displayed a strong blue/black nuclear stain and were counted at ×40 magnification in 3 representative fields per sponge.

Results

Elastase Cleavage of K2P and Purification of Kringle 2

K2P is a bacterially expressed tPA deletion mutant that comprises residues 176 to 527 of native tPA with an additional 3 amino acids Ser-Tyr-Gln at the N terminus.6 To purify the kringle 2 domain, K2P was incubated with increasing concentrations of elastase for 24 hours at room temperature. SDS-PAGE revealed that elastase cleaved K2P into 2 major bands of ≈30 kDa and 10 kDa. Complete cleavage of K2P was obtained at a molar ratio of 10:1 K2P:elastase (Figure 1A, available online at http://atvb.ahajournals.org). N-terminal sequence analysis revealed that elastase cleaved K2P at the Arg^725–He^726 bond, the activation cleavage site of single-chain tPA to 2-chain tPA. The N-terminus of the 30-kDa band was identified as IKGGLF corresponding to residues 276 to 281 of the protease domain of tPA and that of a single-chain tPA to 2-chain tPA. The N-terminus of the 10-kDa band was identified as SYQGN corresponding to the Ser-Tyr-Gln-Gly^176 of native tPA. To confirm cleavage, elastase of 3.5:1 overnight at room temperature with gentle shaking. The digest was extensively dialyzed against column binding buffer (50 mmol/L tris-HCl, 100 mmol/L NaCl, pH 8.4) for 3 hours at 37°C. Absorbance was measured at 405 nm.

In Vitro Cell Proliferation Assays

Cells (3×10^4) were seeded in 96-well plates and allowed to attach overnight. Cells were left in minimal growth medium (M199 with 2% FBS) for 24 hours. Thereafter, reagents were added to wells and incubated for 4 days. Cell number was quantified colorimetrically by the addition of 20 µL CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) and measured at absorbance 492 nm.

Endothelial Cell Migration Assay

Endothelial cell migration was analyzed in a scratch wound assay as previously described with some modifications.21 Endothelial cells (1×10^4) were seeded onto gelatin-coated 3.5-cm dishes and left overnight. Media was aspirated the next day and cells were scratched with a P200 pipette tip along the diameter of the dish. Cells were washed twice with PBS before addition of fragments of tPA in M199 and low serum (5% FBS) and incubated at 37°C and 5% CO2. Wound diameters were photographed at t=0, t=24 hours, and t=48 hours. Wound closure was determined by measuring the diameter of wounds at 3 equivalent points along the length of wounds and was expressed as a percentage of wound diameter at the start of the experiment.

Mouse Sponge Angiogenesis Assay

C57 black mice were implanted with a sterile polymer sponge (15×5×5 mm) under the dorsal skin as previously described.22 PBS, bFGF, or test reagents were injected daily through the skin into the sponge in a volume of 100 µL for 20 days. Sponges were dissected on day 21 and fixed in 3.7% formaldehyde, embedded in paraffin, cut in sections of 4 µm, and stained with hematoxylin and eosin. Morphometric analysis of fibrovascular growth (microvessels and fibroblasts) into sponges was performed as previously described with some modifications.23 A grid graticule of 1-mm² divisions with a total field area of 100 mm² was used to determine the volume fraction of fibrovascular growth contained in sponges.
kinetics, and temperature conditions to effectively separate the kringle 2 domain from the protease domain without compromising the conformational integrity of the kringle 2 module. Final separation of the kringle 2 domain from the protease domain was achieved after a further purification round on lysine-Sepharose. Purity of kringle 2 preparations was confirmed by Western blot with polyclonal anti-tPA IgG (Figure ID, lane 3) and with monoclonal anti-K2 IgG (data not shown).

Fragments of tPA Inhibit Endothelial Cell Proliferation

K2P was incubated with human umbilical vascular endothelial cells (HUVECs) for 4 days at increasing concentrations in the presence of bFGF. Figure 1A shows that K2P significantly decreased bFGF-induced endothelial cell proliferation. Similar results were obtained with HDMEC and BAMEC (data not shown). This effect was specific for endothelial cells because no inhibition of cell growth was observed with vascular smooth muscle cells, normal fibroblasts (MRC-5), or the breast and prostate tumor cell lines MCF-7 and DU145, respectively (Figure 1D). To determine whether the inhibition of bFGF-induced endothelial cell proliferation was dependent on the proteolytic activity of K2P, it was catalytically inactivated so that it was no longer able to activate plasminogen. Catalytically inactivated K2P (K2Pi) also significantly inhibited bFGF-induced HUVEC growth in a similar manner to K2P (Figure 1B), indicating that the proteolytic activity of K2P was not responsible for the inhibition of cell growth observed. The inhibition of bFGF-induced cell growth mediated by K2P and K2Pi was comparable to that of kringle 1 to 4 of plasminogen (angiostatin) (Figure 1C). Figure 1E shows the effects of full-length wild-type rtPA on HUVECs, MCF-7, and DU145 cells for comparison. Inhibition of bFGF-mediated HUVEC proliferation was observed with rtPA, but this was not specific for endothelial cells because a similar degree of inhibition was obtained with both MCF-7 and DU145 cells. This indicates there was no specific endothelial cell inhibition by wild-type rtPA.

To determine whether the kringle domain of K2P was responsible for the inhibition observed, K2P was incubated with HUVECs in the presence of an anti–kringle 2 antibody. Figure 2A shows that the inhibitory activity of K2P was reversed in the presence of anti–kringle 2, indicating that the kringle 2 domain mediated the inhibition of HUVEC proliferation. This was confirmed with the purified kringle 2 domain obtained from elastase cleavage of K2P. Purified kringle 2 significantly inhibited both vascular endothelial growth factor (VEGF)-induced and bFGF-induced HUVEC growth (Figure 2B). Specificity of the kringle 2-mediated effects for endothelial cells was confirmed as no inhibition of K2 was observed with the nonendothelial cell types, human vascular smooth muscle cells, Bowes melanoma cell line, and the human fibrosarcoma cell line HT-1080 (Figure 2C).

Fragments of tPA Inhibit Endothelial Cell Migration

Endothelial cell migration was analyzed in a scratch wound assay. Figure 3A shows the effects of K2P on microvascular...
PMBEC migration. All wound diameters were similar after initial scraping of cells ($t=0$ hour). K2P inhibited cell migration in a time-dependent and concentration-dependent manner. Graphical representation is given in Figure 3B. At 24 hours and 48 hours after cell scraping, statistically significant inhibition of cell migration was observed with 2.5 μmol/L K2P. Figure 3C shows the effects of K2P, K2Pi, and K2 on VEGF-stimulated and bFGF-stimulated HUVEC migration. All fragments of tPA significantly inhibited HUVEC migration to a similar extent.

**Fragments of tPA Inhibit Angiogenesis**

After confirming that both human and mouse endothelial cell migration was inhibited in vitro by kringle 2-containing fragments of tPA, we analyzed their effects in a mouse sponge angiogenesis model in vivo. Purified kringle 2 at 0.1 μmol/L, 1 μmol/L, or 5 μmol/L was administered daily into sponges for a total of 20 days, either in the absence or presence of daily injections of bFGF. Control treated sponges received daily injections of PBS alone or daily injections of bFGF as a positive control. Specific CD34 vessel staining allowed quantification of the number of microvessels contained within sponge sections. The mean number of vessels ± SD determined in 0.1 μmol/L, 1 μmol/L, or 5 μmol/L K2-treated sponges in the absence of bFGF was 7.5±4.7, 4.5±1.3, and 1.5±0.6, respectively, which was statistically significant ($P<0.05$, ANOVA, n=4). A similar trend was observed in the presence of bFGF, where the mean number of vessels ± SD determined in 0.1 μmol/L, 1 μmol/L, or 5 μmol/L K2-treated sponges was 13.8±1.9, 5.3±1.5, and 1.5±1.0, respectively ($P<0.01$, ANOVA, n=4). Figure 4 shows representative hematoxylin and eosin-stained (left panel) and CD34-stained (right panel) sections of sponges treated with PBS, bFGF, or bFGF and 5 μmol/L K2. As well as a significant decrease in bFGF-induced angiogenesis in the sponge assay, it was also observed that the extent of invading mouse stromal tissue that had migrated into the sponge was also inhibited by K2P and purified kringle 2. The volume fraction of fibrovascular growth of K2P-treated sponges was consistently <50% of the total available growth area. Control sponges from mice treated with PBS or bFGF typically
Apoptosis Proliferation

Figure 5. Induction of endothelial cell apoptosis by K2P. TUNEL-stained (left panel) or Ki-67–stained (right panel) sections of sponge at ×40 magnification after administration of PBS (a, b), 2 ng/mL bFGF (c, d), 0.25 μmol/L K2P (e, f), 2 ng/mL bFGF and 0.25 μmol/L K2P (g, h), or 0.25 μmol/L rtPA (i, j). Arrows indicate stained endothelial cells; v indicates vessel. Three mice were used for each treatment.

displayed volume fractions of fibrovascular growth in the region of 70% and 95%, respectively.

We wanted to identify potential mechanisms of action of K2P on angiogenesis inhibition. Kringle domains of plasminogen have been shown to induce apoptosis of endothelial cells. To identify whether K2P could also induce apoptosis in vivo, TUNEL staining was performed on K2P-treated sponges. Representative sections of sponge are given in Figure 5 for TUNEL (left panel) and Ki-67, which was used as a marker of proliferation (right panel). No difference in the number of Ki-67–stained cells was found between K2P-treated sponges and control treated; however, the mean number±SD of apoptotic cells was significantly greater in K2P-treated sponges (34±8) as compared with PBS-treated controls (17±4, P<0.05) (Figure II and Table I, available online at http://atvb.ahajournals.org). We also compared the effects of wild-type rtPA, both in the presence and absence of bFGF, and found no endothelial cell apoptosis in rtPA-treated sponges.

Discussion

The presence of cryptic antiangiogenic domains located within larger molecules that do not possess antiangiogenic activity is an emerging theme in angiogenesis biology. In the hemostatic system of particular interest are the kringle domains that are present within a number of molecules that regulate coagulation and fibrinolysis. We were interested in whether the thrombolytic therapy drug, Reteplose, could also inhibit angiogenesis caused by the presence of the kringle 2 domain. Previous studies have shown that kringle domains do not possess antiangiogenic activity in the intact parent molecule, but only when isolated after enzyme cleavage or expressed as recombinant domains. However, the K2 domain is more exposed in this tPA mutant because of the deletion of the preceding finger and kringle 1 domains. We observed that this drug had very significant effects on endothelial cell proliferation, migration, and angiogenesis. We found that catalytically active K2P and inactivated K2P inhibited bFGF-induced endothelial cell growth to a similar extent. This indicated that inhibition of endothelial cell proliferation was unlikely to be caused by plasminogen activation by K2P, but rather caused by a region located within the K2 domain. This was confirmed after elastase cleavage of K2P and purification of the K2 domain. K2 dose-dependently inhibited both VEGF and bFGF endothelial cell growth. To our knowledge, elastase cleavage of tPA at the Arg373–Ile376 bond has not been reported previously. However, this enzyme has been used extensively to produce fragments of plasminogen and to generate angiostatin. Our data show that elastase can also be used for limited proteolysis of K2P. Reduction of the Cys394–Cys395 disulphide bond was necessary to completely separate the protease domain from the kringle 2 domain. Other studies have shown that selective reduction of disulphide bonds is possible with limited use of DTT. We found that 1 mmol/L DTT was sufficient to selectively reduce the Cys389–Cys385 disulphide bond without compromising the conformational state of the kringle 2 module, as evidenced by the fact that K2 was still able to bind lysine-Sepharose after DTT treatment.

Human and mouse endothelial cell migration was significantly reduced in a scratch wound assay after treatment with K2 containing tPA fragments, as was in vivo angiogenesis in a mouse sponge angiogenesis assay. Of particular interest was the very significant reduction of mouse stromal fibroblasts that had invaded into sponges after tPA fragment treatment. We found no effects of K2P on normal fibroblast proliferation in vitro or Ki-67 proliferating cells in vivo indicating that the reduction of total fibrovascular growth in sponges was not caused by toxicity, but most likely caused by the significant loss of vascularity in the sponges. In support of this are previous studies using disc23 and sponge23 angiogenesis models, which have shown that fibrovascular growth in these models is directly proportional to vessel growth, suggesting a dependency of fibroblast density on the number of blood vessels. In addition, we have also demonstrated increased overall apoptosis in the K2P-treated sponges, both in the absence and presence of bFGF, suggesting a potential mechanism of action of K2P. Although we observed inhibition by wild-type tPA in our assays, this was not specific for endothelial cells and may have been caused by other mech-


anisms, such as proteolytic degradation. That different mecha-
nisms were in operation is supported by the fact that we
found no endothelial cell apoptosis with wild-type tPA in
the sponge assay. This indicates that the antiangiogenic activity is
differentially available in K2P. Figure III (available online at
http://atvb.ahajournals.org) compares the amino acid se-
quence of the K2 domain of tPA with kringle domains of
plasminogen, uPA, and with the kringle 1 domain of tPA. K2
of tPA has the highest identity with K1 of tPA (53%) and with
the kringle domain of uPA (47%). Considerable sequence
identity is also observed with the kringle domains of plas-
minogen (37% to 43%). Despite these similarities, a common
structural motif that might confer the antiangiogenic proper-
ties of kringle domains has not been determined. However, it
cannot be excluded that the antiangiogenic activity is caused
by the secondary structure of these domains in their interac-
tion with the endothelial cell surface.

tPA fibrinolytic drugs are used to treat acute myocardial
infarction, pulmonary embolism, and stroke. However, com-
plications of fibrinolytic therapy include bleeding, of which
intracranial hemorrhage is the most serious. K2P has a
reduced plasma clearance and prolonged half-life compared
with full-length tPA as the finger, epidermal growth factor-
like domain, and the kringle 1 domain, which confer recogni-
tion by clearance receptors, have been deleted. Loss of the
finger domain also results in decreased fibrin specificity of
K2P that can lead to more extensive systemic plasminogen
activation and bleeding. Our data suggest that inhibition of
endothelial cell growth and migration of cells into areas of
damaged endothelium may contribute to bleeding complica-
tions observed during thrombolytic therapy with Retepase.
In addition, our findings suggest that the kringle 2 domain of
tPA may have use as an angiogenic inhibitor, which we are
currently evaluating as a therapeutic target.

Acknowledgments

Funds from Cancer Research UK and charitable donations supported
this work. Our thanks also go to Dr Nick Totty for N-terminal
sequence analysis, to Chris Selkirk for endotoxin assays, and to
George Elia for hematoxylin and eosin staining.

References

1. Carmeliet P, Collen D. Molecular analysis of blood vessel formation and
2. Larsson A, Astedt B. Immunohistochemical localisation of tissue plas-
minogen activator and urokinase in the vessel wall. J Clin Pathol. 1985;
38:140–145.
3. Paciucci R, Tora M, Diaz VM, Real FX. The plasminogen activator
system in pancreas cancer: role of t-PA in the invasive potential in vitro.
V, Brunner G. Urokinase-type and tissue-type plasminogen activators are
5. Llevadot J, Giugliano RP, Antman EM. Bolus fibrinolytic therapy in
plasminogen activator domain-deletion mutant BM 06.022: modular sta-
bility, inhibitor binding, and activation cleavage. Biochemistry. 1994;33:
11760–11766.
7. Cao Y, Cao R, Veitomnaki N. Kringle structures and antiangiogenesis.
Suppression of angiogenesis and tumor growth by the inhibitor K1–5
generated by plasmin-mediated proteolysis. Proc Natl Acad Sci U S A.
HGF/NK4, a four-kringle antagonist of hepatocyte growth factor, is an
angiogenesis inhibitor that suppresses tumor growth and metastasis in mice.
10. Lee T-H, Rhim T, Kim SS. Prothrombin kringle-2 domain has a growth
inhibitory activity against basic fibroblast growth factor-stimulated cap-
SY, Kang D-K, Chang S-I, Chung SI. Anti-angiogenic activity of the
recombinant kringle domain of urokinase and its specific entry into
12. O’Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M,
Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis
inhibitor that mediates the suppression of metastases by a Lewis lung
13. Cao Y, Ji RW, Davidson D, Schuller J, Marti D, Sohndel S, McCanse SG,
O’Reilly MS, Llinas M, Folkman J. Kringle domains of human angio-
5 of plasminogen is a novel inhibitor of endothelial cell growth. J Biol
15. Ji W-R, Castellino FI, Chang Y, DeFord ME, Gray H, Villarreal X,
Eghtedarzadeh Kon dri M, Marti D, DN, Llinas M, Schaller J, Kramer RA,
Trail PA. Characterization of kringle domains of angiostatin as antago-
nists of endothelial cell migration, an important process in angiogenesis.
S, Schnaper HW, Pizzo SV. Angiostatin binds ATP synthase on the
surface of human endothelial cells. Proc Natl Acad Sci U S A. 1999;96:
2811–2816.
17. Troyanovsky B, Levchenko T, Mansson G, Matvijenko O, Holmgren L.
Angiostatin: an angiostatin binding protein that regulates endothelial cell
BKL, Wu Z, Grau GE, Shing Y, Soff GA, Bouck N, Pepper MS. Multiple
forms of angiostatin induce apoptosis in endothelial cells. Blood. 1998;
92:4730–4741.
B, Cao Y. Endothelial cell surface ATP synthase-triggered caspase-apo-
ptotic pathway is essential for k1–5–induced antiangiogenesis. Cancer
Y-K, Joe YA. Inhibition of endothelial cell proliferation by the recombi-
nant kringle domain of tissue-type plasminogen activator. Biochem
21. Cattaruzza S, Schiappacassi M, Ljungberg-Rose A, Spessotto P,
Perissinotto D, Morinelli M, Micugnan MT, Colombani A, Perris R.
Distribution of PG-M versus variants in human tissues and de novo
expression of isoform V3 upon endothelial cell activation, migration, and
23. Hu DE, Hiley CR, Snither RL, Gresham GA, Fan TP. Cor relation of
133Xe clearance, blood flow and histology in the rat sponge model for
angiogenesis. Further studies with angiogenic modifiers. Int J Bio-
24. Mulradihara BK, Hirose M. Structural and functional consequences of
removal of the interdomain disulfide bridge from the isolated C-lobe of
25. Koziolowski J, Kwan HH, Pronas SD, Allison AC, Fajardo LF. Character-
ization and applications of the disc angiogenesis system. Exp Mol Pathol.
Antiangiogenic Activity of a Domain Deletion Mutant of Tissue Plasminogen Activator Containing Kringle 2
Veronica A. Carroll, Leonid L. Nikitenko, Roy Bicknell and Adrian L. Harris

Arterioscler Thromb Vasc Biol. 2005;25:736-741; originally published online February 3, 2005; doi: 10.1161/01.ATV.0000157980.15710.2b

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/4/736

Data Supplement (unedited) at:
http://atvb.ahajournals.org//subscriptions/

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplementary Data

Anti-angiogenic activity of Reteplase: inhibition mediated by the kringle 2 domain.

Materials
Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were purchased from R&D Systems. Anti-kringle 2 antibody (7VPA) was purchased from Technoclone. D-Phe-Pro-Arg-chloromethylketone (PPACK) was obtained from Calbiochem. Phenylmethylsulfonyl fluoride (PMSF) and porcine pancreatic elastase were purchased from Roche. Angiostatin was obtained from Technoclone.

Cell Culture
Human umbilical vein endothelial cells (HUVEC) and human vascular smooth muscle cells (HVSMC) were purchased from Technoclone and maintained in endothelial cell culture medium, M199 with Earle’s salts (Sigma) containing 20% heat inactivated fetal bovine serum (Sigma), 100 IU/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), 250 ng/mL fungizone (Gibco), 2 mM glutamine (Gibco), 5 IU/mL heparin (Sigma) and 50 µg/mL endothelial cell growth supplement (Sigma). Human dermal microvascular endothelial cells (HDMEC) were cultured in EGM-MV BulletKit (Clonetics) according to manufacturer's instructions. Bovine adrenal microvascular endothelial cells (BAMEC) were purchased from VEC Technologies, Inc. and cultured according to manufacturer’s instructions. Primary mouse brain endothelial cells (PMBEC) were cultured in endothelial cell culture medium as above. Normal human fibroblasts, MRC-5, and the
human tumor cell lines, MCF-7 (breast), DU145 (prostate), Bowes (melanoma), and fibrosarcoma (HT-1080) were all maintained in DMEM supplemented with 10% FBS.
Fig. I. Purification of K2 domain of tPA.

(A) K2P was incubated with increasing concentrations of elastase overnight at room temperature to cleave the kringle 2 domain (K2) from the protease domain (P) and the digests were subjected to 15% SDS-polyacrylamide gels and stained with Coomassie blue. Molecular weight markers (lane 1), K2P (lane 2), K2P incubated with elastase at ratios of 100:1 (lane 3), 10:1 (lane 4), 2:1 (lane 5), 1:1 (lane 6). (B) 15% SDS-polyacrylamide gel. Molecular weight markers (lane 1), K2P (lane 2), K2P after elastase digestion (lane 3), K2P after elastase digestion and dialysis with column binding buffer (lane 4), eluted material following lysine-Sepharose chromatography (lane 5), elastase (lane 6). (C) Samples in panel B were subjected to SDS-PAGE before Western blotting with anti-elastase IgG. (D) Western blot with polyclonal anti-tPA IgG. K2P (lane 1), elastase cleaved K2P (lane 2), K2 following DTT treatment and affinity purification (lane 3).
Fig. II. Graphical representation of TUNEL staining.

Data represent the average number of apoptotic cells ± SD. Three mice were used for each treatment. *p<0.05 Student’s t-test.
Table I. Quantification of angiogenesis, apoptosis and cell proliferation in vivo.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vessels*</th>
<th>TUNEL†</th>
<th>Ki-67‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>11 ± 3</td>
<td>17 ± 4</td>
<td>80 ± 9</td>
</tr>
<tr>
<td>bFGF</td>
<td>17 ± 5</td>
<td>13 ± 2</td>
<td>109 ± 11</td>
</tr>
<tr>
<td>K2P</td>
<td>6 ± 3</td>
<td>34 ± 8</td>
<td>76 ± 2</td>
</tr>
<tr>
<td>bFGF+K2P</td>
<td>12 ± 5</td>
<td>32 ± 5</td>
<td>101 ± 10</td>
</tr>
<tr>
<td>rtPA</td>
<td>5 ± 4</td>
<td>15 ± 5</td>
<td>115 ± 11</td>
</tr>
</tbody>
</table>

* Total number of vessels per field of view ± SD
† Total number of TUNEL positive cells per field of view ± SD
‡ Total number of Ki-67 positive cells per field of view ± SD
Fig. III. Amino acid sequence alignment of kringle 2 of tPA with other kringle domains of the hemostatic system.

Comparison of the amino acid sequence of the kringle 2 domain of tPA with other anti-angiogenic kringle domains which were aligned according to their conserved cysteines.