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In vitro and in vivo antiangiogenic activity of a novel deca-peptide derived from human tissue-type plasminogen activator kringle 2 ☆

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A synthetic deca-peptide corresponding to the amino acid sequence Arg^{54}-Trp^{63} of human tissue-type plasminogen activator (t-PA) kringle 2 domain, named TKII-10, is produced and tested for its ability to inhibit endothelial cell proliferation, migration, tube formation in vitro, and angiogenesis in vivo. At the same time, another peptide TKII-10S composed of the same 10 amino acids as TKII-10, but in a different sequence, is also produced and tested. The results show that TKII-10 potently inhibits VEGF-stimulated endothelial cell migration and tube formation in a dose-dependent, as well as sequence-dependent, manner in vitro while it is inactive in inhibiting endothelial cell proliferation. Furthermore, TKII-10 potently inhibits angiogenesis in chick chorioallantoic membrane and mouse cornea. The middle four amino acids DGDA in their sequence play an important role in TKII-10 angiogenesis inhibition. These results suggest that TKII-10 is a novel angiogenesis inhibitor that may serve as a prototype for antiangiogenic drug development.

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1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is involved not only in various physiological processes but also in many human diseases such as tumor growth and metastasis, diabetic retinopathy, and inflammation. Excessive angiogenesis always leads to further deterioration in these diseases [1]. Angiogenesis is a multi-step process that includes endothelial cell proliferation and migration, capillary tube formation, and extracellular matrix degeneration and remodeling. The process of angiogenesis is tightly regulated by the balance between angiogenic stimulators and angiogenic inhibitors. As previous reports confirm that vascular endothelial growth factor (VEGF) is the key factor in stimulating angiogenesis, more and more attention is directed at the study of angiogenic inhibitors [2,3].

To date, some angiogenic inhibitors derived from various endogenous proteins have been reported to inhibit angiogenesis in vitro and vivo [4,5]. The recombinant protein, TK1-2, consisted of tissue-type plasminogen activator (t-PA) kringles 1 and 2 (Ala^{90}-Thr^{263}) has been reported to inhibit endothelial cell proliferation, migration, tube formation, and angiogenesis in vivo [6–8]. In addition, reteplase, the thrombolytic therapy drug comprised of the kringle 2 and the protease domain of t-PA (K2P) has also been reported to inhibit endothelial cell proliferation and migration in vitro, and angiogenesis in vivo as well. The authors identify the kringle 2 domain of t-PA as a novel target for antiangiogenic therapy [9]. Kringle domains are protein modules composed of about 78–80 amino acids connected by a characteristic triple disulfide-linked loop [10]. Apart from the previous reports on TK1-2 and K2P, some recent studies also have shown that isolated kringle domains of endogenous proteins involved in the hemostatic system are antiangiogenic [11,12].

Comparing to the proteins with kringle domains, small peptides have advantages for therapeutic applications due to their high solubility, increased bioavailability and lack of immune response in the host cell. Furthermore, production of a peptide is less difficult and more controllable than production of a protein. Therefore, designing and developing peptides for therapeutic application to inhibit angiogenesis is an important area in antiangiogenic drug development [13].

In this study, a novel deca-peptide named TKII-10, representing the Arg^{54}-Trp^{63} amino acid of human t-PA kringle 2, was synthesized and tested for its antiangiogenic activities in vitro and vivo. We identified that TKII-10 functioned as a potent angiogenic inhibitor, inhibiting VEGF-stimulated endothelial cell migration and capillary tube formation in a dose-dependent and sequence-dependent manner, while it was ineffective in inhibiting VEGF-stimulated endothelial cell proliferation. In addition, relevant mechanisms of inhibition are discussed.

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2. Materials and methods

2.1. Peptide synthesis

Using the cysteine in disulfide bonds for cleavage sites (not including cysteine), human t-PA kringle 2 domain was mainly separated into four peptides. This paper focused on one of these four peptides, representing the Arg^{54}-Trp^{63} amino acid of human t-PA kringle 2, which was named TKII-10. The amino acid composition of TKII-10 was RNPDGDAKPW. In order to identify whether the antiangiogenic effect of TKII-10 was sequence-dependent and to determine the key amino acid sequence of TKII-10 function, we scrambled the amino acid sequence of TKII-10 and synthesized several peptides, including TKII-10S (RNPDAGDKPW), which was set as a contrast peptide in all the following experiments in vitro and in vivo. At present, the antiangiogenic effects of the other three peptides derived from human t-PA kringle 2 are under investigation in our laboratory.

The solid-phase synthesis of TKII-10 peptide was performed by ChinaPeptides Co., Ltd. in Shanghai, PR China with a high-efficiency solid-phase method using an automatic peptide synthesizer (Symphony; Protein Technologies, Tucson, AZ). The peptide was characterized by high-performance liquid chromatography (HPLC, analytical; Shimadzu, Kyoto, Japan) and mass spectrometry (MS, Finnigan TSQ 7000; Thermo, Waltham, MA), and then freeze-dried and stored at −20 °C until used.

2.2. Materials

Human $VEGF_{165}$ was purchased from R&D Systems Inc. (Minneapolis, MN). Gelatin, poly-HEMA, sucrose octasulfate–aluminum complex were purchased from Sigma–Aldrich (St. Louis, MO). Transwell chamber (8.0-μm pore size) was obtained from Costar (Corning, Cambridge, MA). Growth factor reduced Matrigel was obtained from BD Biosciences (Bedford, MA). Human umbilical vein endothelial cells (HUVECs) and endothelial cell culture media ECM were obtained from ScienCell Research Laboratories (San Diego, CA). HUVECs were cultured at 37 °C in a humidified 5% CO_2 atmosphere. All cells used for the experiments were from passages 3 to 8.

2.3. Endothelial cell migration assay

To determine the effect of TKII-10 peptide on HUVECs migration toward VEGF, an endothelial cell migration assay was performed using a disposable Transwell chamber as described previously with modifications [14]. Using the Transwell chamber, the membrane was coated with 0.1% gelatin. Briefly, about 4×10^5 HUVECs were pre-incubated with various concentrations of peptide at 37 °C for 30 min before being seeded onto the gelatin-coated cell culture inserts. VEGF (25 ng/ml) was placed into the lower chamber. The assembled cell culture insert chamber was then incubated at 37 °C for 24 h. After removing the non-migrating cells with a cotton swab, migrated cells on the lower surface of the culture inserts were fixed with 4% paraformaldehyde, stained with hematoxylin, and photographed under a light microscope. Five random fields were chosen in each insert, and the cell number was counted. All the experiments were performed in triplicate.

2.4. Endothelial cell tube formation assay

Growth factor reduced Matrigel (50 μl) was added to each well of chilled 96-well plates and incubated for 30 min at 37 °C. About 3×10^4 HUVECs were pre-incubated with various concentrations of peptide at 37 °C for 30 min before being seeded onto the solidified growth factor reduced Matrigel in a 96-well plate. After incubating in media with or without 15 ng/ml VEGF at 37 °C for 6 h, tube formation was observed under a light microscope and photographed. Four random fields were chosen in each well, and the total tube length was quantified by using NIH ImageJ 1.32 software.

2.5. Endothelial cell proliferation assay

A cell proliferation assay was determined by using the non-radioactive CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) kit (Promega, Madison, WI). Briefly, approximately 3500 HUVECs were added in triplicate into each well of 96-well cell culture plates and incubated at 37 °C for 24 h. Then HUVECs were starved overnight with ECM containing 0.5% FBS. HUVECs were incubated in ECM plus 0.5% FBS with or without 10 ng/ml VEGF and various concentrations of peptide for 24 h. Then 20 μl MTS solution was added to each well and incubated for another 3 h at 37 °C. The absorbance of A490 nm, which correlates to the number of living cells, was measured with a microplate reader (Bio-Rad; Model 680; USA).

2.6. Chick chorioallantoic membrane assay

To determine antiangiogenic activity in vivo, a chorioallantoic membrane (CAM) assay was performed as previously described with modifications [15]. Two-day-old fertilized eggs (Shanghai Poultry Breeding Co. Ltd.. China) were incubated at 37 °C and 60–70% relative humidity. After 5 days of incubation, a 1–2 cm^2 window was opened and a sterile round filter paper (5 mm in diameter, Whatman qualitative filter papers; Sigma–Aldrich, St. Louis, MO) containing phosphate-buffered saline (PBS) or peptide (10 or 50 ng/filter paper) was applied onto the CAM of every individual embryos. After another 2 days of incubation, the upper eggshell was removed and capillaries within 2.5 mm around the filter paper were observed and photographed under a stereomicroscope (Olympus, SZX16).

2.7. Mouse corneal micropocket assay and histological examination

In order to further evaluate the antiangiogenic efficacy of TKII-10 in vivo, a mouse corneal micropocket assay was performed according to procedures previously described [15]. Five- to six-week-old, age-matched C57BL/6 male mice (Shanghai Laboratorial Animal Center, Chinese Academy of Sciences) were used in this study. The animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All of the experimental protocols were approved by the Animal Investigation Committee of Shanghai First People's Hospital, Affiliate of Shanghai Jiaotong University. The sustained release micropellets were made of the slow-release hydron polymer (poly-hydroxyethylmethacrylate, poly-HEMA) containing sucralfate, VEGF (160 ng per pellet), and/or different dose of peptide (1 or 5 μg per pellet).

Briefly, C57BL/6 mice were randomly assigned to each experimental group ($n = 8$ for each group) and the right eye of each mouse was used for the operation. Paracentral (1.0 mm from the limbus), intrastromal linear keratotomy was performed in the topically anesthetized eyes, and an approximate 0.5 mm × 0.5 mm micropocket was dissected parallel with the limbus. Then, a single slow-release pellet was placed into the pocket in each eye. The corneal neovascularization was examined and photographed using a stereomicroscope (Olympus, SZX16) 5 days after pellet implantation. The length and clock hours of new blood vessels were measured. The area of neovascularization was calculated according to the formula: area (mm^2) = $0.2 \times 3.14 \times$ vessel length (mm) $\times$ clock number $\times$ 0.4 (mm). Then, mouse corneas were dissected and fixed in 10% formaldehyde. The paraffin embedded corneal tissues was

sectioned in 3 μm slices and subjected to hematoxylin and eosin staining for histological analysis.

2.8. Statistical analysis

The experimental data were expressed as mean ± SEM from at least three independent experiments. Analysis was performed with one-way ANOVA for multiple variables and with Student's *t*-tests for comparison of two groups with normal distribution using the statistical software program SPSS13.0 for Windows (Chicago, IL). *P* values <0.05 were considered significant in all cases.

3. Results

3.1. TKII-10 inhibits VEGF-stimulated endothelial cell migration and tube formation

The inhibitory effect of TKII-10 peptide on VEGF-stimulated migration of HUVECs was evaluated in a disposable Transwell chamber. TKII-10 potently inhibited VEGF-stimulated HUVECs migration in a dose-dependent, as well as sequence-dependent, manner with an estimated ED_{50} value between 1 μM and 10 μM, whereas TKII-10S had no such activity even at 10 μM (Fig. 1A). We also examined the effect of the TKII-10 peptide on tube formation of HUVECs on Matrigel. As shown in Fig. 1B, TKII-10 effectively inhibited VEGF-stimulated HUVECs tube formation on Matrigel dose-dependently between 100 nM and 10 μM. TKII-10 also inhibited HUVECs tube formation sequence-dependently as TKII-10S did not demonstrate any inhibitory effect on HUVECs tube formation even up to the concentration of 10 μM.

3.2. TKII-10 does not inhibit VEGF-stimulated endothelial cell proliferation

Although TKII-10 effectively inhibited VEGF-stimulated HUVECs migration and tube formation, it demonstrated no inhibitory effect on VEGF-stimulated HUVECs proliferation. In the endothelial cell proliferation assay, HUVECs were exposed to increasing concentrations of TKII-10 peptide for 24 h in the presence of 10 ng/ml VEGF as a mitogenic stimulus. As shown in Fig. 2, VEGF effectively stimulated HUVECs proliferation compared with the no-VEGF group. TKII-10 and TKII-10S did not show any obvious inhibitory effect on HUVECs proliferation up to the concentration of 10 μM at 24 h.

3.3. TKII-10 inhibits angiogenesis in the chick embryo

The chick chorioallantoic membrane assay was used to investigate the effect of TKII-10 and TKII-10S on angiogenesis in vivo. As shown in Fig. 3, most of chick chorioallantoic membranes in the PBS group did not demonstrate any obvious avascular zone around

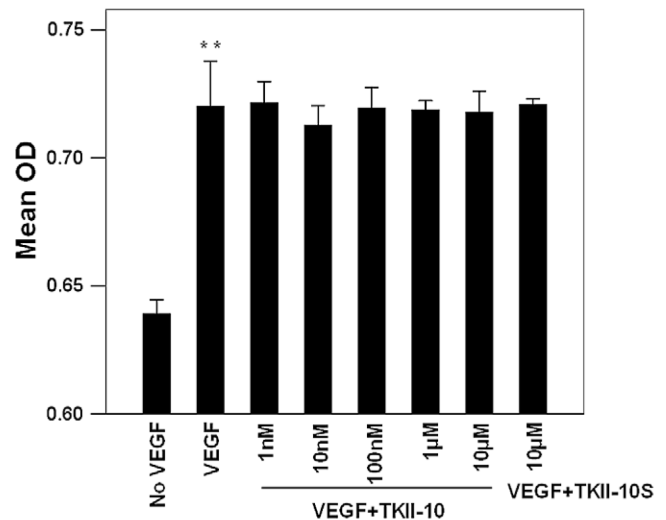

Fig. 2. Effects of TKII-10 on VEGF-stimulated HUVECs proliferation. Inhibitory effect of TKII-10 on endothelial cell proliferation was assessed by MTS method 24 h after treatment. Each value represents the mean ± SEM of triplicate measurements. **$P < 0.01$ versus the no-VEGF group.

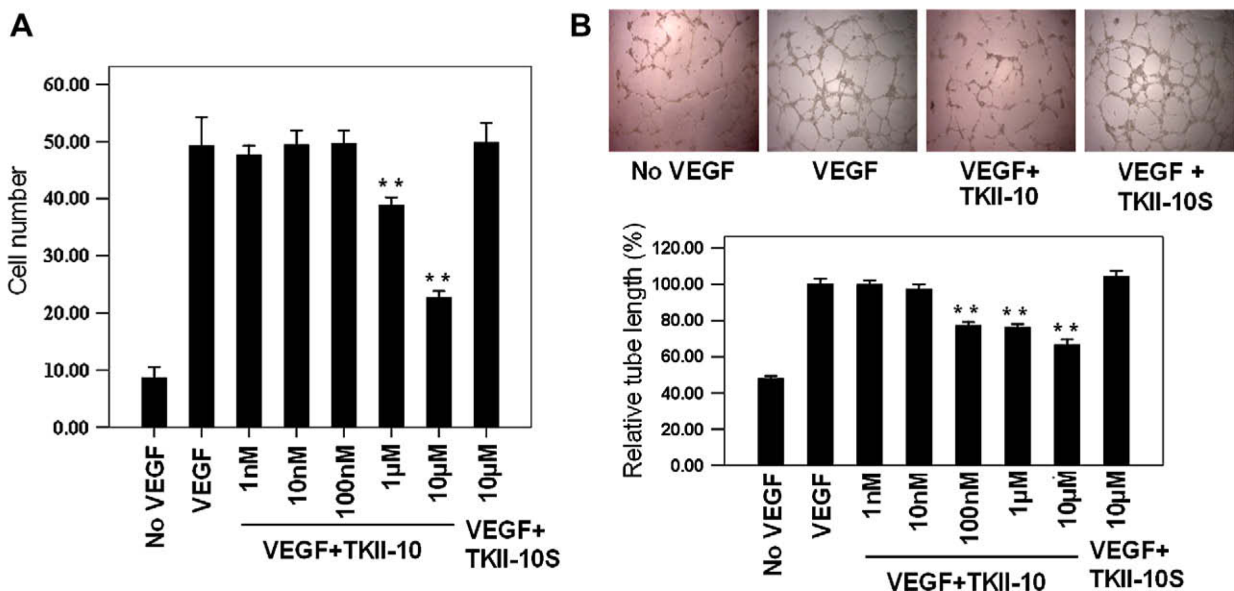

Fig. 1. Effects of TKII-10 on VEGF-stimulated HUVECs migration and tube formation on Matrigel in vitro. (A) Inhibitory effect of TKII-10 on endothelial cell migration toward VEGF was assessed by a Transwell chamber 24 h after treatment. (B) The upper panel is the representative phase-contrast micrographs of HUVECs tube formation on Matrigel exposed to culture medium (without VEGF and TKII-10), VEGF (15 ng/ml), VEGF plus TKII-10 (10 μM), and VEGF plus scrambled peptide TKII-10S (10 μM) at 6 h (magnification 20×). The lower panel presents the quantitative graph of the relative percentage of tube length from TKII-10 plus VEGF treatment group compared with the VEGF-only group. Each value represents the mean ± SEM of triplicate measurements. **$P < 0.01$ versus the VEGF-only group.

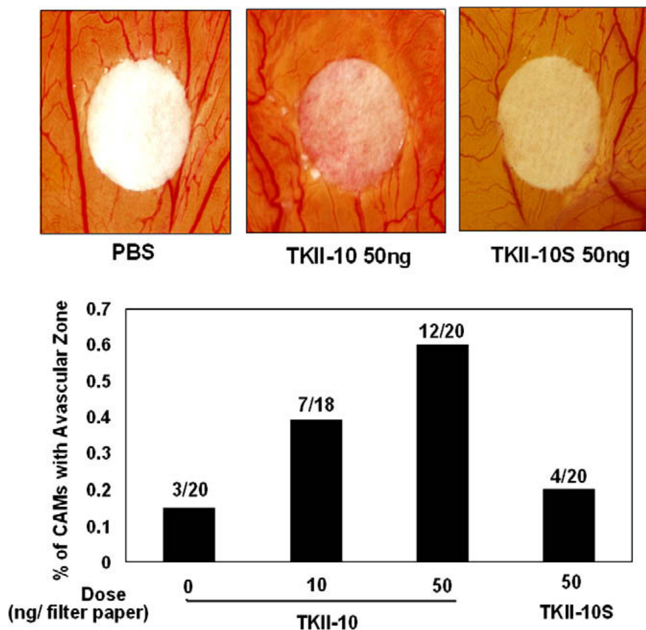

Fig. 3. Inhibition of angiogenesis in chick chorioallantoic membranes by TKII-10. Filter papers containing variable amounts of TKII-10 were applied to the CAMs. After 48 h, the avascular zone (2.5 mm around the filter paper) of each CAM was examined. The upper panel shows the representative appearances of chick CAMs implanted with a filter paper containing no TKII-10 (PBS; left), 50 ng TKII-10 (middle), and 50 ng TKII-10S (right) (magnification 10×). The lower graph represents the inhibition (%) of treated samples, which is presented as the number of CAMs with avascular zones over the total number of CAMs (indicated above each column).

the implanted filter paper. However, TKII-10 inhibited the development of new embryonic capillaries and produced an avascular zone around the implanted filter paper. Most of chick chorioallantoic membranes in the TKII-10S group did not produce an avascular zone round the implanted filter paper. TKII-10 inhibited chick embryonic angiogenesis in a dose-dependent manner from 10 to 50 ng/embryo whereas TKII-10S had no such activity even at 50 ng/embryo. No obvious toxicity was observed in the embryos used in the experiments.

3.4. TKII-10 inhibits VEGF-induced angiogenesis in mouse cornea

To further investigate the antiangiogenic activity of the TKII-10 peptide in vivo, the inhibitory effect of TKII-10 on VEGF-induced corneal neovascularization was examined in the mouse corneal angiogenesis assay (Fig. 4). Implantation of pellets containing VEGF-induced obvious corneal new blood vessel formation (Fig. 4A). In contrast, implantation of TKII-10 or PBS pellets did not induce neovascularization in mouse corneas. However, implantation of pellets containing VEGF plus TKII-10 resulted in significantly reduced corneal neovascularization compared with that in eyes implanted with VEGF pellets only (Fig. 4B and C), whereas implantation of pellets containing VEGF plus TKII-10S demonstrated obvious corneal neovascularization (Fig. 4D). Corneal histological analysis revealed that new blood vessels formed in the corneal stroma in the VEGF pellet implantation group (Fig. 4E). TKII-10 significantly reduced new blood vessel formation in corneal stroma, without overt cytotoxicity to other corneal cells (Fig. 4F and G). However, in TKII-10S plus VEGF pellet implantation corneas, obvious new blood vessels formed in the stroma (Fig. 4H). Moreover, TKII-10 inhibited VEGF-induced corneal neovascularization dose-dependently. At day 5 post-operation, TKII-10 significantly decreased the length (Fig. 4I), clock hours (Fig. 4J), and area (Fig. 4K) of VEGF-induced blood vessels whereas TKII-10S had no such activities. Together, these results strongly supported that TKII-10 effectively inhibited angiogenesis in vivo.

4. Discussion

Although t-PA kringle 1-2 and kringle 2 alone are reported to inhibit growth factors induced endothelial cell proliferation, migration, and angiogenesis in vivo [6–9,16,17], the functional element in these domains is still not clear. Previous data have shown that human t-PA kringle 2 domain has the highest identity with kringle 1 of t-PA, and considerable sequence identity with kringle domains of urokinase plasminogen activator (uPA) and plasminogen [9], which have been identified as angiogenesis inhibitors in other studies. Therefore, we chose t-PA kringle 2 as the sample protein for antiangiogenic peptide derivation and the antiangiogenic molecular mechanism study.

We showed that a human t-PA kringle 2 derived deca-peptide inhibited VEGF-stimulated different aspects of angiogenesis. As we all know, VEGF is the most important angiogenic stimulator that induces all necessary components of an angiogenic response. Since TKII-10 inhibits VEGF-stimulated endothelial cell migration and tube formation in vitro, TKII-10 is expected to be a useful angiogenic inhibitor. We further demonstrated that TKII-10 potently inhibited chick CAM capillary development and VEGF-induced mouse corneal angiogenesis in vivo.

Although purified human t-PA kringle 2 significantly inhibits both VEGF-induced and bFGF-induced HUVECs growth [9], the TKII-10 peptide, which derives from t-PA kringle 2, demonstrates no obvious inhibitory effect on VEGF-stimulated endothelial cell proliferation. Interestingly, there is a similar case in our laboratory. A novel peptide KV11, which consists of 11 amino acid residues from human apolipoprotein(a) kringle 5-like domain, inhibits VEGF-induced migration and tube formation of HUVECs, without any anti-proliferative effect, either [18]. One explanation of these features is that the remaining amino acids apart from the derived peptide in t-PA kringle 2 or apolipoprotein(a) kringle 5 may contribute to the proliferation inhibitory function. Nevertheless it cannot be excluded that the anti-proliferative activity of these kringle domains may be caused by a secondary structure in their interaction with the endothelial cell surface, not only by certain amino acids.

TK1-2, from which TKII-10 is derived, is reported to inhibit endothelial cell migration through inhibition of signaling and cytoskeleton rearrangement in part by interfering with integrin $\alpha2\beta1$ [19], and the molecular mechanisms of this interference could be further elucidated by our study. Integrin $\alpha2\beta1$, which provides critical support for VEGF signaling, endothelial cell migration, and tumor angiogenesis [20,21], is one of the major receptors for the extracellular matrix collagen I. The DGEA (Asp-Gly-Glu-Ala) sequence within collagen I is the major recognition site for integrin $\alpha2\beta1$ [22], which is similar to the DGDA (Asp-Gly-Asp-Ala) sequence within TKII-10. A hypothesis of the present study is that this DGDA sequence plays an important role in inhibiting endothelial cell migration and tube formation, because it may compete with collagen type I in binding integrin $\alpha2\beta1$. To prove that, we design the TKII-10S peptide, the sequence of which only differs from TKII-10 peptide in the middle four amino acids—DAGD and DGDA. As the following experiments find that this TKII-10S has no antiangiogenic activity, it provides strong evidence of the previous hypothesis. While our experiments were ongoing, Kim and Joe reported results of a study on a synthesized tetra-peptide DGDA [23]. They found that DGDA peptide inhibited adhesion of HUVECs to immobilized TK1-2 and pretreatment of the DGDA peptide also blocked anti-migratory activity of TK1-2. When the DGDA peptide alone was tested for antiangiogenic activity in vitro, it effectively

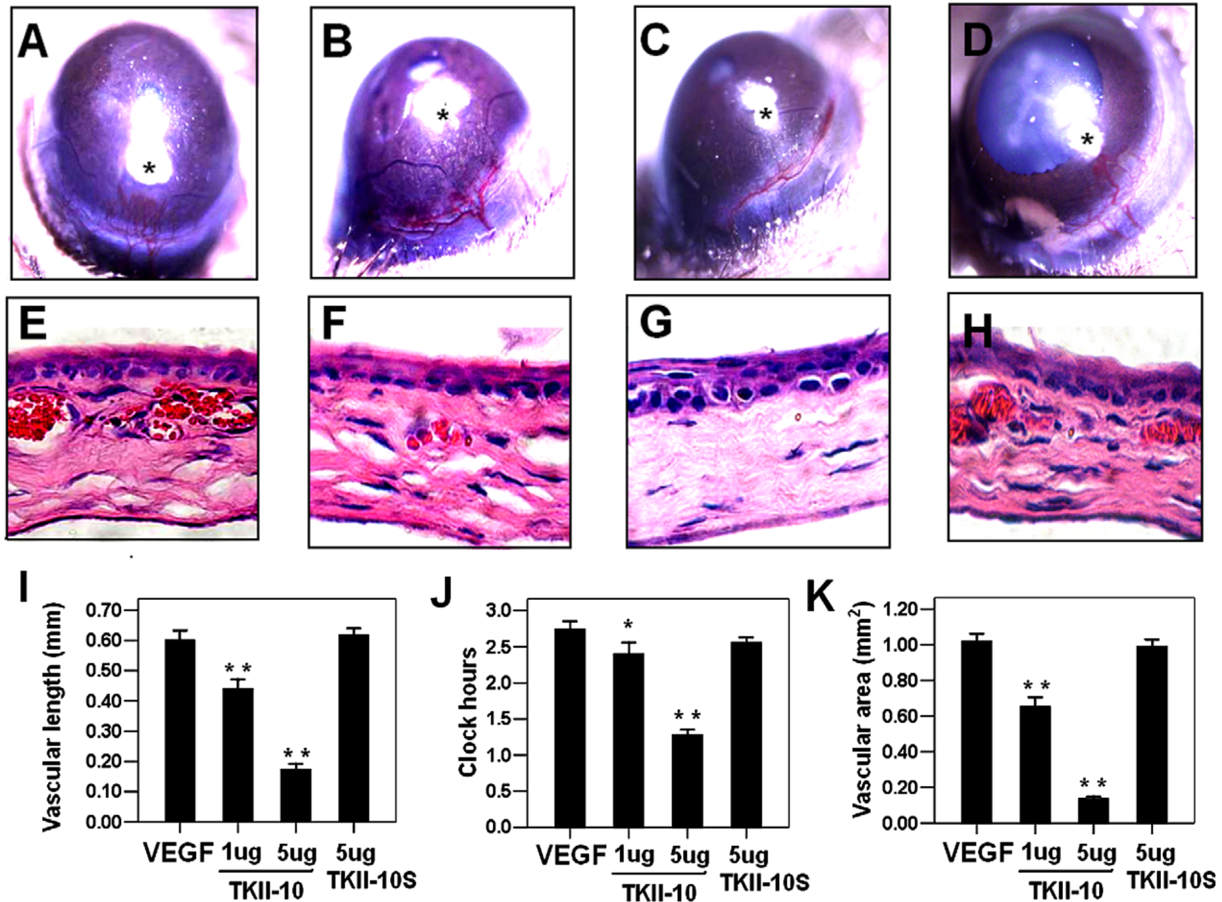

Fig. 4. Inhibition of VEGF-induced corneal neovascularization by TKII-10. (A–D) Representative mouse cornea photographs after implantation of a VEGF pellet (160 ng per pellet; A), a pellet containing VEGF plus TKII-10 (160 ng for VEGF and 1 μg for TKII-10 per pellet; (B) a pellet containing VEGF plus TKII-10 (160 ng for VEGF and 5 μg for TKII-10 per pellet; (C) or a pellet containing VEGF plus TKII-10S (160 ng for VEGF and 5 μg for TKII-10S per pellet; (D) for 5 days. The asterisk represents the place of hydron pellet implantation. (E–H) Histological analysis of neovascularization in mouse corneas after implantation VEGF pellets, pellets containing VEGF and TKII-10 or pellets containing VEGF and TKII-10S (100×). In VEGF pellet implantation eyes, multiple lumen-like formations containing red blood cells presented in the corneal stroma. The surrounding stroma exhibited edema (E). In VEGF plus TKII-10 1 μg pellets implantation eyes, fewer new capillaries were observed (F). There was no significant neovascularization shown in eyes receiving VEGF plus TKII-10 5 μg pellets implantation (G). In VEGF plus TKII-10S 5 μg pellets implantation eyes, obvious lumen-like formations were observed in the corneal stroma (H). (I–K) The length (I), clock hours (J), and area (K) of neovascularized vessels in mouse corneas were measured after implantation for 5 days. Bars represent the means and the error bars represent the SEM. $^*P < 0.05$ versus the VEGF-only group. $^{**}P < 0.01$ versus the VEGF-only group.

inhibited VEGF-induced migration of HUVECs and tube formation on Matrigel. Our in vitro studies on TKII-10 have similar results and, furthermore, our in vivo studies strongly support that the DGDA sequence presents a functional epitope of t-PA kringle 2.

Peptide pharmaceuticals are progressively making their way into clinical applications. Several peptides with antiangiogenic activities have been obtained from various domains of endogenous proteins [18,24,25]. Peptide-based drug therapy provides a promising alternative for angiogenesis-related diseases. Although the peptide may not inhibit all the complex multi-step procedures of angiogenesis, suppression at any one of the angiogenic steps will inhibit the formation of new vessels and lead to the disruption of angiogenesis. These peptides can serve as potential candidates for therapeutic intervention of angiogenesis. We demonstrate here a novel antiangiogenic peptide TKII-10, and the advantages of TKII-10 peptide, compared to TK1-2 and K2P, are simplicity and convenience in production (chemical synthesis by solid-phase methods), viability for modifications in chemical synthesis, lower-antigenicity, higher water solubility, and improved bioavailability over large proteins [26].

In summary, the human t-PA kringle 2 derived peptide TKII-10 effectively inhibits angiogenesis in vitro and in vivo. The middle four amino acids DGDA in their sequence play an important role in TKII-10 angiogenesis inhibition. This TKII-10 peptide may act as prototype for further development of antiangiogenic drugs.

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