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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

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