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Enhanced Anti-Angiogenic Effect of a Deletion Mutant of Plasminogen Kringle 5 on Neovascularization

Weibin Cai,[1] Jianfang Ma,[2] Chaoyang Li,[3] Zhonghan Yang,[1] Xia Yang,[1] Wei Liu,[4] Zuguo Liu,[3] Mintao Li,[4] and Guoquan Gao[1]*

[1]Department of Biochemistry, Zhongshan Medical School, Sun Yat-sen University, Guangzhou 510089, Guangdong Province, China
[2]Department of Internal Medicine, 476 Hospital of PLA, Fuzhou 350002, Fujian Province, China
[3]Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510089, Guangdong Province, China
[4]Proteomics Laboratory, Zhongshan Medical School, Sun Yat-sen University, Guangzhou 510089, Guangdong Province, China

Abstract Kringle 5 (K5), a proteolytic fragment of plasminogen, has been proved to be an angiogenic inhibitor. Previously, we have evaluated the effect of K5 on the vascular leakage and neovascularization in a rat model of oxygen-induced retinopathy. In this study, we expressed K5 and a deletion mutant of K5 (K5 mutant) in a prokaryocyte expression system and purified them by affinity chromatography. K5 mutant was generated by deleting 11 amino acids from K5 while retaining the three disulfide bonds. The anti-angiogenic activity of intact K5 and K5 mutant were compared in endothelial cells and retinal neovascularization rat model. K5 mutant inhibited the proliferation of primary human retinal capillary endothelial cells (HRCEC) in a concentration-dependent manner, with an apparent EC_{50} of approximate 35 nmol/L, which is twofold more potent than intact K5. In the even higher concentration range, K5 mutant did not inhibit pericytes from the same origin of HRCEC, which suggested an endothelial cell-specific inhibition. K5 mutant had no effect on normal liver cells and Bel7402 hepatoma cells even at high concentration range either. Intravitreal injection of the K5 and mutant in the oxygen-induced retinopathy rat model both resulted in significantly fewer neovascular tufts and nonperfusion area than controls with PBS injection, as shown by fluorescein angiography. Furthermore, K5 mutant exhibited more strong inhibition effect on neovascularization than intact K5 by quantification of vascular cells. These results suggest that this K5 deletion mutant is a more potent angiogenic inhibitor than intact K5 and may have therapeutic potential in the treatment of those disorders with neovascularization, such as solid tumor, diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, and hyperplasia of prostate. J. Cell. Biochem. 96: 1254–1261, 2005.

Key words: plasminogen kringle 5; angiogenic inhibitors; mutation; pathologic neovascularization

Neovascularization, abnormal formation of new vessels from pre-existing capillaries, is a characteristic pathologic finding of some disorders such as solid tumor [Jimenez and Volpert, 2001], diabetic retinopathy [Hamanaka et al., 2001], rheumatoid arthritis [Koch, 2003], and hyperplasia of prostate [Franck-Lissbrant et al., 1998]. A common mechanism for neovascularization is the disturbed regulation of angiogenesis, which is controlled by a delicate balance between angiogenic stimulators (e.g., vascular endothelial growth factor (VEGF)) and angiogenic inhibitors (e.g., pigment epithelium-derived factor (PEDF)) [Gao et al., 2001; Jimenez and Volpert, 2001]. Stimulators are increased while angiogenic inhibitors are decreased under the above-mentioned pathological conditions [Gao et al., 2001]. These changes

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*Correspondence to: Guoquan Gao, Department of Biochemistry, Zhongshan Medical School, Sun Yat-sen University, Guangzhou 510089, Guangdong Province, China. E-mail: gaog@gzsums.edu.cn

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break the balance in angiogenesis control and consequently result in over proliferation of capillary endothelial cells and abnormal formation of new blood vessels [Kontogeorgos and Kontogeorgou, 2003]. Although the molecular mechanism leading to neovascularization is presently uncertain, some angiogenic inhibitors such as angiostatin [O'Reilly et al., 1994a], PEDF [Dawson et al., 1999], endostatin [O'Reilly et al., 1997], Kallikrein-binding protein [Gao et al., 2003] etc. show intensive anti-angiogenic activity. So angiogenic inhibitor may hold great therapeutic potential in the treatment of neovascular diseases.

Plasminogen kringle 5 (K5), a proteolytic fragment of plasminogen, was discovered to have powerful anti-angiogenic activity [Cao et al., 1997]. Plasminogen contains 5 kringle domains and several of the kringles have displayed anti-angiogenic activities [Castellino and McCance, 1997]. K5 has been shown to inhibit proliferation of endothelial cells [Cao et al., 1997]. Moreover, its inhibitory activity is more potent than angiostatin, which has 98% homogeneity with kringles 1–4 of plasminogen [Cao et al., 1996, 1997]. In addition, K5 also inhibits endothelial cell migration, an important process in angiogenesis [Ji et al., 1998]. Recently, K5 has been found to inhibit retinal neovascularization and thus, may have therapeutic potential in the treatment of diabetic retinopathy such as retinopathy of prematurity (ROP) and solid tumor [Zhang et al., 2001, 2004]. K5 also exerts its effect on endothelial cells by inducing cell cycle arrest and apoptosis and is specific to proliferating endothelial cells [Lu et al., 1999]. Because of its high efficacy, cell type-selectivity, and short amino acid sequence, K5 has considerable potential in the treatment of neovascular diseases [Cao et al., 1997; Cai et al., 2004].

In contrast to its significance, however, the structural basis and mechanism of action for K5 have not been elucidated [Cai et al., 2004]. K5 is a proteolytic fragment with the sequence of plasminogen Pro^{452}-Ala^{542}. It contains an intact kringle domain (Cys^{462}-Cys^{541}) formed through three disulphide bonds and two arms (Pro^{452}-Asp^{461} and Ala^{542}) outside of the kringle structure [Cao et al., 1997]. Kringle domain is proposed to be responsible for the interaction of plasminogen with the corresponding effector molecules, such as fibrinogen, fibrin, and thrombospondin. It has been demonstrated that the kringle structure is essential in maintaining the potent anti-endothelial functions of angiostatin. Recently, some data show that reducing and alkylating K5 could significantly increase its anti-migratory activity, suggesting that some sequences of K5 may shield the functional elements of K5 from effectively interacting with endothelial cells. The two amino acid arms of K5 may be considerably responsible for the "shielding effect." To define the minimal peptide sequence required for the anti-angiogenic activity and get a new angiogenic inhibitor, the present study generated a deletion mutant of K5 and compared its activity with intact K5 in endothelial cell and in the oxygen-induced retinopathy rat model.

MATERIALS AND METHODS

Reagents, Plasmid, and Bacteria Strain

The recombinant plasmid, pET-22b(+)/K5 was constructed, expressed, purified, and analyzed as described previously [Zhang et al., 2001; Gao et al., 2002b]. The expressing vector of pET-22b(+) and *E. coli* strain BL21(DE3) were purchased from Novagen Co., Madison, WI. The restriction endonuclease *Eco*RI, *Hind*III, and T4 DNA ligase were purchased from New England Biolabs, Ipswich, MA. The His-Bind column was from Novagen Co. and the antibody specific to His-tag was from Oncogene Co., Cambridge, MA. The primers synthesis and DNA sequencing were performed by Sangon Co., Shanghai, China. The enhanced chemiluminescene (ECL) system for Western analysis was purchased from Pierce Co., Rockford, IL.

Generation of Deletion Mutant

The cDNA encoding deletion mutant K5 was generated by polymerase chain reaction (PCR) using the intact K5 cDNA as the template. The 5′ PCR primer (5′-ATGAATTCGTGTATGTTTGGGAATGGG-3′) and the 3′ primer (5′-GCCAAGCTTACACTGAGGGACATCACAGTAG-3′) contain an *Eco*RI and *Hind*III sites, respectively, which can facilitate cloning. The PCR deleted 10 residues from the N-terminus and 1 residues from the C-terminus of K5. The PCR product was cloned into the pET22-b(+) vector at the *Eco*RI and *Hind*III sites in frame with the signal peptide sequence at the 5′ end and the sequence encoding 6 × His at its 3′end. The pET22-b(+)/K5 mutant was identified

by endonuclease digestion and sequenced to ensure PCR fidelity and correct reading frame.

Prokaryotic Expression and Purification of Recombinant Proteins

The pET22-b(+)/K5 or pET22-b(+)/K5 mutant construct was introduced into *E. coli* strain BL21(DE3). This vector provides a signal peptide that enables the recombinant protein to enter the periplasmic space. The expression and purification followed the protocol recommended by Novagen with some modifications. Briefly, expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) and carried out for 10 h at 25°C. Periplasmic proteins were released by digestion with lysozyme and separated from cells by centrifugation. The recombinant peptide was purified by passing through the His-Bind column and dialyzed with high-concentration glycerol buffer (phosphate buffer solution (PBS), pH 7.4, 50% glycerol, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA)). The purity and identity of recombinant peptide was examined by 16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis using an antibody specific to His-tag as described previously [Zhang et al., 2001]. The quantitative measurement of purity and content was processed by thin-layer protein scanning of SDS–PAGE gel with Genegenius gel image system (Gene Co., England).

Molecule Mass Analysis

Matrix assisted laser desorption ionization-time of flight mass spectra (MALDI-TOF MS) were recorded in the linear mode on a Vision 2.01.03 (ETTAN MALDI-TOF Pro, Amersham Biosciences, Sweden) equipped with a 337 nm nitrogen laser. The matrix was cinnamylic acid (Sigma Co., St. Louis, MO) dissolved in 0.1% trifluoroacetic acid at 10 g/L. To determine the protein molecular mass, 1 μl of sample was mixed with 1 μl of matrix and air-dried on the stainless steel probe tip. Mass spectra were acquired by signal averaging a minimum of 200 spectra for each sample. The MALDI-TOF MS system parameters were initially set by using standard proteins with well-characterized molecular masses that theoretically spanned the entire dynamic range of expected results. The mass/charge (m/z) scale was calibrated from the ions generated (from both singly and doubly charged protein species) with these known protein standards.

Cell Culture

Human retinal capillary endothelial cells (HRCEC) and pericytes were isolated from donor eyes, obtained from Zhongshan Ophthalmic Center of SUN Yat-sen University, China, following a protocol described previously [Grant and Guay, 1991]. The identity of HRCEC was confirmed by a characteristic cobblestone morphology and the incorporation of acetylated low-density lipoprotein labeled with a fluorescent probe, DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate) (Biomedical Technologies, Inc., Stoughton, MA) [Grant and Guay, 1991]. Purity of the pericyte culture was determined by immunostaining using a fluorescein isothiocyanate (FITC)-conjugated antibody specific to α-smooth-muscle actin (Sigma Co.).

The liver cell line and hepatocarcinoma cell line, Bel7402 were provided by Cell Bank of China Science Academy. The cells were maintained in RMPI 1640 medium (Gibco BRL, Carlsbad, CA) supplement with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) at 37°C in a 5% CO_2 atmosphere.

Quantification of Viable Cells

Cells were plated in gelatin-coated 12-well plates at a density of 10^4/well in triplicate, respectively and cultured in the growth medium until they reached 60–70% confluency. The culture medium of HRCEC and pericyte was replaced with a medium containing 1% FBS while the culture medium of liver cell and Bel7402 was replaced with a medium containing no FBS. Different concentrations of K5 or mutant were added to the cover medium. The viable cells were quantified after 72 h incubation by the 3-[4, 5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromid (MTT) assay (Roche Co., Germany) following a protocol recommended by the manufacturer. The effect of the K5 or mutant on viable cell number was analyzed by using Student's *t* test.

Induction of Retinal Neovascularization and Intravitreal Injection of K5 or Mutant

In vivo the anti-angiogenic activity of K5 and mutant was detected in a rat model with retinal neovascularization. The rat model of oxygen-induced retinopathy (OIR) was induced as

described by Gao et al. [2002a] with some modifications. Briefly, newborn pigmented Brown Norway rats from Beijing (China) at postnatal day 7 (P7) were randomly assigned to each experimental group and exposed to hyperoxia (75% O_2) for 5 days and then normoxia to induce retinal neovascularization. Animals were anesthetized, and the purified K5 or mutant was injected into the vitreous of the right eye through the pars plana using a glass capillary at P12. The left eye received the same volume of PBS as the control. To compare the effect of K5 and K5 mutant directly, they were injected into the vitreous of different eye in the same rat of OIR with the dose of 3 μg/eye, respectively. After injection, the animals were kept in normoxia for another 5 days for analyses of retinal neovascularization (P17).

Retinal Angiography With High Molecular Weight Fluorescein

Retinal angiography was as described by Gao et al. [2002]. Briefly, altogether three rats per group were anesthetized and perfused with fluorescein via intra-ventricle injection of 50 g/L of high molecular weight (2×10^6) fluorescein isothiocyanate (FITC)- dextran (Sigma Co.). The animals were immediately sacrificed, and the eyes were enucleated and fixed in 4% paraformaldehyde for 10 min. The retina was dissected free of the lens and vitreous and incubated in 4% paraformaldehyde for 3 h. The retina was cut and flat-mounted on a gelatin-coated slide. The vasculature was then examined under a fluorescent microscope (Axioplan2 Imaging, Carl Zeiss, Germany).

Quantification of Preretinal Nuclei

Retinal neovascularization was quantified by counting pre-retinal vascular cells as previously described [Zhang et al., 2001]. Eyes were fixed with 10% formaldehyde, sectioned, and then stained with hematoxylin and eosin. The pre-retinal vascular cells were counted using light microscopy (250×). A total of eight sagittal section from each eye were examined and cell numbers were averaged in each group of animals. The average number of pre-retinal vascular nuclei was compared with the PBS control group by Student's *t* test.

RESULTS

Generation, Expression, and Purification of K5 Deletion Mutant

The K5 mutant was expressed in *E. coli* and purified to apparent homogeneity with the His-bind affinity column. The purified recombinant protein showed an apparent molecular weight of 14 kDa according to Coomassie-stain SDS–PAGE (Fig. 1A). Accurate molecular weight of K5 and mutant obtained by MALDI-TOF MS analysis was 14.9 kDa and 13.7 kDa, respectively (Fig. 2), matching the calculated molecular weight from the sequence. The identity of the band was confirmed by Western blot analysis using an anti-His tag antibody (Fig. 1B). The recombinant protein accounted for about 0.13 g/g of the total bacterial proteins as judged by Coomassie-stain SDS–PAGE scanning of protein. At least 90% purity of recombinant K5 and mutant was achieved by metal-chelating resin

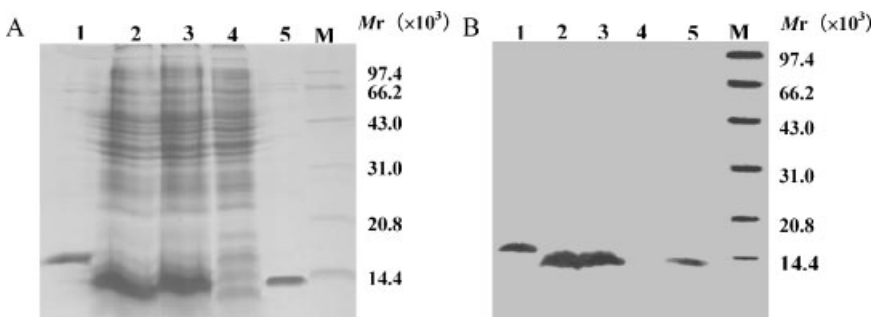

Fig. 1. Expression and purification of recombinant peptide. **A**: SDS–PAGE with Coomassie blue staining. **B**: Western blot analysis with an antibody specific to the His-tag. **Lane 1**: Affinity-purified K5; (**lanes 2**, **3**) crude extract after the IPTG induction; (**lane 4**) crude cell extract before the IPTG induction; (**lane 5**) affinity-purified K5 mutant; M, protrein marker.

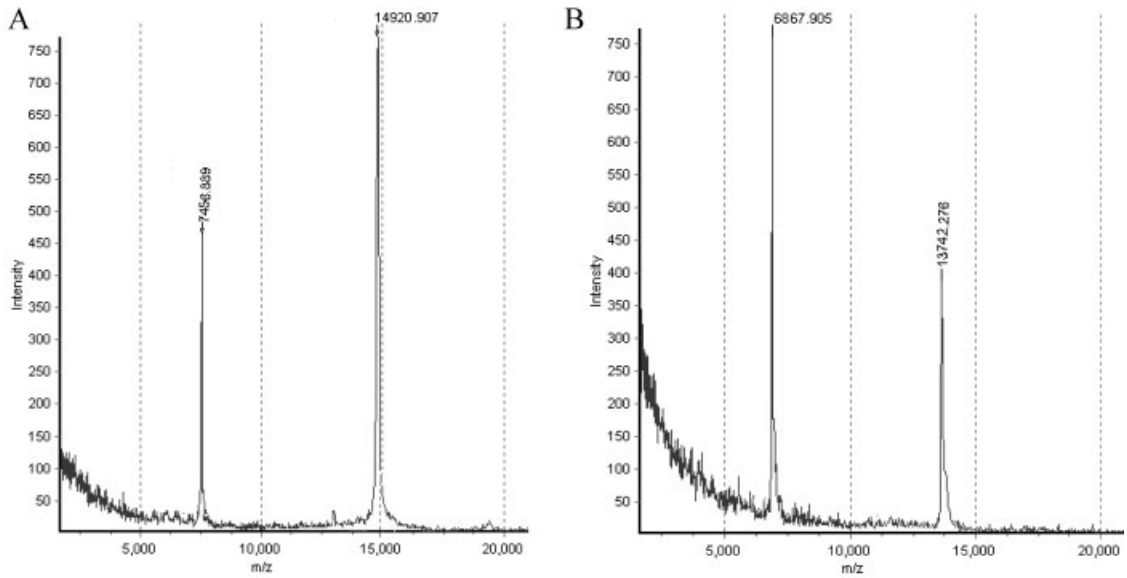

Fig. 2. MALDI-TOF mass spectra for purified K5 and K5 mutant. The m/z range (x-axis) was 0–20,000 with the apparent molecular masses of the highest-intensity ion species identified. **A**: MALDI-TOF mass spectra for K5. **B**: MALDI-TOF mass spectra for K5 mutant.

(Ni^{2+}-TNA) affinity chromatography (IMAC) approach and glycerol dialysis. An average of 10–20 mg of purified peptide was obtained from 1 L of culture.

More Potent Inhibition of Endothelial Cell Proliferation by K5 Mutant

HRCEC were treated with the recombinant K5 mutant at concentrations of 5, 10, 20, 40, and 80 nmol/L for 72 h. The viable cells were quantified by MTT assay. At a concentration as low as 10 nmol/L, the K5 mutant treatment resulted in significantly fewer viable cells than the control cells ($P < 0.05$). This effect appeared to be K5 mutant concentration-dependent (Fig. 3A). Even in higher concentration range, the K5 mutant did not result in any significant inhibition of pericytes from the same origin as the HRCEC, liver cells, and Bel7402 cells, suggesting endothelial cell-specific inhibition (Fig. 3B). The mutant showed an EC_{50} of approximately 35 nmol/L while intact K5 has an EC_{50} of approximately 70 nmol/L in inhibiting HRCEC proliferation (Fig. 3A). The deletion mutant, compared with intact K5, has an enhanced anti-angiogenic activity.

Enhanced Anti-Angiogenic Effect of the K5 Mutant in an OIR Model

Retinal neovascularization was induced in Brown Norway rats by exposure of newborn rats to hyperoxia as described previously [Gao et al., 2001]. The K5 mutant was injected intravitreally (3 μg/eye) at P12 after the return of the animals from hyperoxia to normoxia, and the control eyes received the same volume of PBS. Rats were kept under normoxia for another 5 days and the retinal neovascularization was examined by fluorescein angiography (P17). The control eyes (PBS-injected) developed typical retinal neovascularization, including neovascular tufts, microaneurysms, enlarged non-perfusion regions, and vascular leakage (Fig. 4A,C). As compared to the control group, the eyes injected with the K5 mutant showed an apparent improvement in retinal vasculature and reduction in neovascular tufts (Fig. 4B). The K5 intravitreal injection group also showed less neovascularization compared with the control group, but the extent looked not as apparently as K5 mutant injection group (Fig. 4C,D).

Quantification of pre-retinal neovascular cells demonstrated that the K5 mutant at 3 μg/eye significantly decreased pre-retinal vascular cells (25.7 ± 3.1 pre-retinal cells/section, $P < 0.05$, $n = 8$) while the lower dose (1 μg/eye) had no significant inhibition (92.3 ± 4.7 pre-retinal cells/section, $n = 8$) as compared with the control eye treated with PBS (Fig. 5A). This result suggests that a single injection of this peptide inhibits retinal neovascularization under ischemic conditions and this inhibition effect is

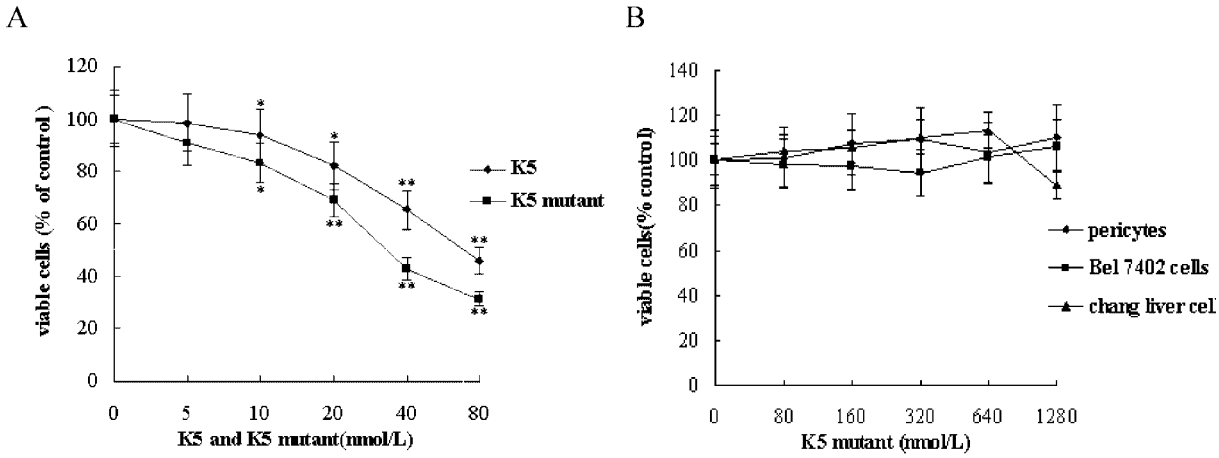

Fig. 3. Specific inhibition of endothelial cells. Cells were treated with the recombinant K5 and K5 mutant at concentrations as indicated for 72 h. The viable cells were quantified using the MTT assay. Values represent absorbance as percentages of respective controls. Each data is expressed as the mean ± SE of six determinations in three separate experiments. The 0 groups without K5 or K5 mutant treatment are used as controls. **A**: Effect of K5 and K5 mutant on the proliferation of primary HRCEC. **B**: Effect of K5 mutant on the proliferation of pericytes, Bel7402 cells and chang liver cells (*$P < 0.05$, **$P < 0.01$ *vs.* control).

dependent on the concentration of K5 mutant. These results suggested that K5 mutant, in comparison with intact K5 (43.5 ± 4.9 pre-retinal cells/section, $P < 0.05$, n = 8), also has an enhanced anti-angiogenic activity in the animal model (Fig. 5B). These results were further confirmed by injection of K5 and K5 mutant in the same rats at different eye (Fig. 5C).

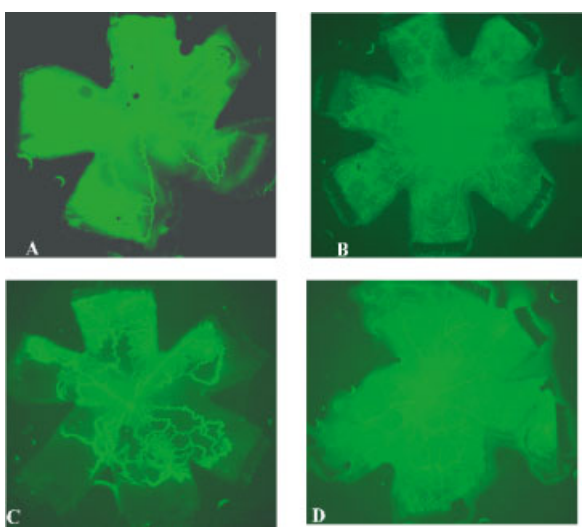

Fig. 4. Inhibition of ischemia-induced retinal neovascularization. Retinal neovascularization was induced in newborn Brown Norway rats. Retinal vasculature was examined by angiography at 5 days after the injection of K5, K5 mutant or PBS (control). **A**: Left retina from OIR rat after PBS injection as the control of the K5 mutant-treated group. **B**: Right retina from OIR rat after the K5 mutant injection. **C**: Left retina from OIR rat after PBS injection as the control of the K5-treated group. **D**: Right retina from OIR rat after K5 injection. Each image is a representative from three animals of each group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Since the disturbed balance between angiogenic stimulators and inhibitors is the common mechanism leading to neovascularization, it is hypothesized that delivery of a potent angiogenic inhibitor should prevent or arrest the process of neovascularization. In the past few years, a number of endogenous angiogenic inhibitors have been identified, such as PEDF, maspin, anti-thrombin III [Dawson et al., 1999; O'Reilly et al., 1999; Zhang et al., 2000], endostatin, angiostatin, K5, tumstatin, and Kallikrein-binding protein [O'Reilly et al., 1994b, 1997; Cao et al., 1996, 1997; Maeshima et al., 2001; Gao et al., 2003]. Among them, K5 is one of the most potent angiogenic inhibitors [Cao et al., 1997]. In this study, we used the methods of gene mutation and genetic engineering to get K5 mutant, which deleted 11 amino acids from the side arms of K5, and demonstrated that the deletion mutant actually has more powerful anti-angiogenic activity than K5. As shown in the Figure 3A, this deletion improved the anti-angiogenic activity in vitro by approximately twofold over the intact K5, that is, the K5 mutant showed an EC_{50} of approximate 35 nmol/L while intact K5 has an EC_{50} of approximate 70 nmol/L in inhibiting of HRCEC proliferation. Moreover, in the oxygen-induced retinopathy model, K5 mutant also

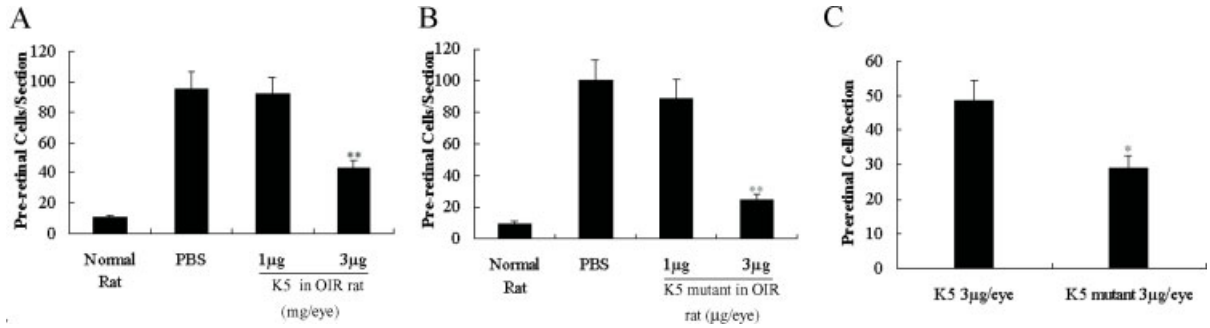

Fig. 5. Decrease of pre-retinal vascular cells in OIR rats. Pre-retinal vascular cells were counted on sagittal sections from the animals 5 days after the injection of K5, K5 mutant or PBS (control). **A**: Pre-retinal vascular cell count after K5 and PBS injection ($n = 8$, $^{**}P < 0.01$ *vs.* PBS group). **B**: Pre-retinal vascular cell count after K5 mutant and PBS injection ($n = 8$, $^{**}P < 0.01$ *vs.* PBS group). **C**: Pre-retinal vascular cell count after K5 and K5 mutant injection in the same rats at at different eye ($n = 5$, $^{*}P < 0.05$ *vs.* K5 group). Bars represent cell average numbers per section.

showed enhanced anti-angiogenic activity compared to K5.

It was previously reported that exposure of the hidden active element in K5 with reducing and alkylating agents could enhance the anti-angiogenesis activity [Ji et al., 1998]. K5 is a proteolytic fragment with the sequence of plasminogen Pro^{452}-Ala^{542} and contains an intact kringle structure and two amino acid arms [Cao et al., 1997]. This study deleted both the arms and just left the intact kringle domain with three disulphide bond to form K5 mutant (Cys^{462}-Cys^{541}). The results show that this deletion improves the anti-angiogenic activity and suggests that the arms outside the disulphide bonds may not be essential for the anti-angiogenic activity. The present observations lead to the proposal that the two arms sequence may shield the functional elements of K5 from effectively interacting with endothelial cells. This may possibly explain why K5 mutant has higher activity than K5. On the other hand, deletion of the two arms may change the conformation of kringle domain. So although K5 mutant and K5 have the same sequence of kringle domain, they may have different structure. Kringle structures have been proved to play an important role in maintaining the potent anti-endothelial function of angiostatin [Cao et al., 1996]. But it is still uncertain about the relation of kringle structure and anti-angiogenic activity of K5 [Cai et al., 2004]. It would be of great value to investigate the kringle domain integrity and the biological activity of K5.

The potential therapeutic effect of K5 in the treatment of ocular neovascular diseases such as diabetic retinopathy and retinopathy of prematurity (ROP) has been explored extensively [Gao et al., 2002]. For our knowledge, its application in the treatment of cancer has not been reported yet. Our recent experiments indicated that K5 and mutant exhibit anti-tumor activity in a xenografted hopatocarcinoma mouse model (data not shown). However, as shown in Figure 3, K5 mutant have no apparent inhibition of hopatocarcinoma cell proliferation. So the present study suggests that the anti-angiogenic activities of K5 and mutant may be the main mechanism responsible for the anti-tumor effect.

As shown in Figure 4, a single injection of the K5 mutant only showed a partial inhibition of retinal neovascularization. This is possibly due to the rapidly decreasing concentration of the peptide in the vitreous. Improving the availability of this peptide by novel delivery techniques, such as a sustained-releasing pellet or gel, or gene delivery, will be indispensable to achieve prolonged availability of an angiogenic inhibitor in the vitreous after a single dose injection [Ozaki et al., 1997].

No detectable inhibition of normal liver cells and no apparent histological evidence of retinal toxicity were observed after K5 mutant injection, which suggested that this peptide, at the concentrations used, do not cause any detectable toxicity to the normal liver and to the retina. In contrast to intact K5, K5 mutant has high stability and is suitable for a sustained-releasing system because it has a short peptide sequence and simple conformation. Moreover, in this study, we demonstrate that deletion of 11 amino acids from the side arms of the K5 actually possesses more potent inhibitory activity on neovascularization than intact K5 both

in vitro and in vivo. These properties make this peptide a promising candidate for therapeutic applications in the treatment of neovascular diseases such as solid tumor, diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, and hyperplasia of prostate.

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