Nanoparticle-mediated Expression of an Angiogenic Inhibitor Ameliorates Ischemia-induced Retinal Neovascularization and Diabetes-induced Retinal Vascular Leakage

Short Title: Nanoparticles for diabetic retinopathy

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Submitted 27 September 2008 and accepted 4 May 2009.

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**Objective:** The objective of the present study is to evaluate the effect of nanoparticle-mediated gene delivery of angiogenic inhibitors on retinal inflammation, vascular leakage and neovascularization in diabetic retinopathy.

**Research design and methods:** An expression plasmid of plasminogen kringle 5 (K5), a natural angiogenic inhibitor, was encapsulated with poly lactide-co-glycolide to form K5 nanoparticles (K5-NP). Expression of K5 was determined by Western blot analysis and immunohistochemistry. Retinal vascular leakage was measured by permeability assay. Retinal neovascularization was evaluated using fluorescein-angiography and counting pre-retinal vascular cells in rats with oxygen-induced retinopathy (OIR). Effects of K5-NP on retinal inflammation were evaluated in streptozotocin (STZ)-induced diabetic rats by leukostasis assay and Western blot analysis of ICAM and VEGF. Possible toxicities of K5-NP were evaluated using histology examination, retinal thickness measurement, and electroretinogram (ERG) recording.

**Results:** K5-NP mediated efficient expression of K5 and specifically inhibited growth of endothelial cells. An intravitreal injection of K5-NP resulted in high-level expression of K5 in the inner retina of rats for the entire 4 weeks analyzed. Injection of K5-NP significantly reduced retinal vascular leakage and attenuated retinal neovascularization, when compared to the contralateral eyes injected with Control-NP in OIR rats. K5-NP attenuated VEGF and ICAM-1 over-expression, reduced leukostasis and vascular leakage for at least 4 weeks after a single injection in the retina of STZ-induced diabetic rats. No toxicities of K5-NP were detected to retinal structure and function.

**Conclusion:** K5-NP mediates efficient and sustained K5 expression in the retina and has therapeutic potential for diabetic retinopathy.
Retinal vascular leakage and neovascularization are the major features of diabetic retinopathy and the leading causes of vision loss (1-3). These retinal vascular abnormalities are also common in other ocular disorders such as sickle cell retinopathy, retinal vein occlusion and retinopathy of prematurity (1; 4; 5). Vascular endothelial growth factor (VEGF) is known to play a key pathogenic role in the blood-retinal barrier (BRB) breakdown or vascular leakage and retinal neovascularization (6-8).

Angiogenesis is regulated by two counter-balancing systems between angiogenic stimulators, such as VEGF, and angiogenic inhibitors, such as angiostatin (4; 9; 10). Angiostatin contains the first four triple disulfide bond-linked loops of plasminogen known as kringle domains and is a potent inhibitor of angiogenesis (11). Among proteolytic fragments of plasminogen, kringle 5 (K5), an 80-amino acid peptide from plasminogen, has the most potent inhibitory effect on endothelial cell growth (12). Previously, we have shown that K5 inhibits ischemia-induced retinal neovascularization in the oxygen-induced retinopathy (OIR) model (13). K5 also reduces retinal vascular leakage in the OIR model and in the streptozotocin (STZ)-induced diabetes model (14). The K5-induced reduction of vascular leakage can be achieved through an intraocular, periocular, topical or systemic administration of the K5 peptide (15). Similar to that of many other anti-angiogenic peptides, however, these K5 effects are transient after a single injection of the K5 peptide due to its short half-life in the retina (14; 15). A sustained ocular delivery of K5, such as gene therapy, is desirable for the development of a long-term treatment of diabetic retinopathy.

Traditionally, gene delivery systems can be classified into viral vector-mediated and non-viral deliveries. Currently, viral vectors are the most commonly used means for gene delivery, due to their high efficiencies (16; 17). The limitations of viral vector-mediated delivery, such as potential risks, restricted targeting of specific cell types, and immunogenecity of viral vectors hamper their clinical application (18; 19). For these reasons, non-viral systems for gene delivery have become increasingly desirable in both basic research and clinical settings.

One of the emerging non-viral vector-mediated gene delivery systems is condensation of plasmid DNA or oligonucleotides into nanoparticles (20). Currently, there are several biocompatible polymers to be used for DNA delivery, such as poly D, L-lactide-co-glycolide (PLGA) and poly (ethylene-co-vinyl acetate) (EVAc). Several groups have successfully encapsulated naked DNA into biodegradable PLGA nanoparticles for long-term and controlled DNA release (21). Although matrix-type nanoparticles have been formulated using different polymers, the nanoparticles formulated from PLGA are especially of interest for gene delivery due to their safety, biocompatibility, biodegradability and sustained release characteristics (22; 23).

Herein, we encapsulated an expression plasmid of K5 with PLGA polymer to form nanoparticles and evaluated the efficacy of these K5-nanoparticles (K5-NP) on ischemia-induced retinal vascular leakage and retinal neovascularization in the OIR rat model. We evaluated the effects of K5-NP on retinal inflammation in STZ-induced diabetic rats. In addition, we also tested the ocular toxicities of K5-NP.

**RESEARCH DESIGN AND METHODS**

**Construction of expression vector for K5:** The human K5 cDNA (362 bp) was amplified by PCR using a pair of primers...
containing a 6× histidine tag (His-tag) at the C-terminus of K5. For secretion of K5, a 52-bp linker encoding the signal peptide (SP) was cloned into a pcDNA3.1(+) vector at the BamHI and XbaI sites, in frame with the SP sequence. The resulting pcDNA3.1(+)–SP-K5-6×His-tag expression construct was confirmed by restriction digestion and DNA sequencing.

Preparation and characterization of PLGA:CHN-K5 nanoparticles:

Preparation of poly (lactide-co-glycolide) PLGA:Chitosan pK5 nanoparticles: PLGA:Chitosan nanoparticles containing the K5 expression plasmid were prepared using a previously reported emulsion-diffusion-evaporation technique (24) with some modifications. Briefly, 15.5 mg of PLGA (50:50, i.v. 0.17, Birmingham Polymers Inc.) was dissolved in 5 mL of ethyl acetate. The PVA solution (1%, w/v) was prepared and then chitosan chloride (Nova Matrix PCL 113, 2.5 mg) was dissolved with stirring. Plasmid solution (1 mL containing 350 µg of plasmid) was modified with 10% (w/v) sodium sulfate and added to the chitosan solution for complexation and DNA condensation with stirring. Both solutions were then combined and emulsified with a probe sonicator for 4 min. To the emulsion, approximately 30 mL of Milli-Q H2O was added and stirred on a magnetic plate stirrer for 3 h to evaporate the solvent. The particle suspension was ultracentrifuged, resuspended in Milli-Q H2O, and the procedure was repeated twice. Upon final resuspension in Milli-Q H2O the nanoparticle suspension was lyophilized to obtain dry particles.

Particle size and zeta-potential measurement: The particle size and size distribution were determined using dynamic light scattering (Brookhaven Instruments Corp., Holtsville, NY). The same equipment was used to determine the zeta-potential of the particles.

Estimation of plasmid loading in nanoparticles: For plasmid loading estimation, the lyophilized product (0.2 mg) was dispersed in 1 mL of methylene chloride to dissolve the polymer, followed by extraction of the plasmid into 2 mL of Tris-EDTA (TE) buffer. An aliquot of the TE buffer fraction was analyzed for the absorbance at 260 nm to determine the plasmid content per mg of nanoparticles.

Western blot analysis: For in vitro assay, protein extracts were prepared from a human Müller cell line (MIO-M1) (a generous gift from Drs. Khaw and Limb (25)) treated with Control-NP or K5-NP under hypoxia for 48 h. For in vivo assay, the free-floating retinas were homogenized in 150 µL of ice-cold tissue lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM MgCl2, 1% NP-40, 10% glycerol, 100 mM NaCl), and a protease inhibitor cocktail (1:100 dilution, Sigma-Aldrich, St. Louis, MO) added just before use. The lysates were cleared by centrifugation at 12,000×g for 20 min at 4°C. The protein concentration of the lysate was determined using the Bradford assay. The same amount of proteins (50 µg) was resolved by SDS-PAGE in 8% denaturing gels and transferred onto nitrocellulose membranes. Ponceau S staining (Sigma-Aldrich) was carried out to verify equal protein loading. Immunoblotting and signal detection by ECL were performed as described previously by incubating the membranes with the primary antibodies against rabbit anti-VEGF (1:1000 in 5% non-fat milk powder/0.1% TBS Tween-20, Santa Cruz), HIF-1α (1:1000; BD Transduction Laboratories), goat anti-ICAM-1 (1:1000; Santa Cruz), mouse anti-His-tag (1:2000; Upstate), mouse anti-TATA box-binding protein (TBP,1:1000; Abcam), or mouse anti-β-actin (1:2000; Abcam) antibodies overnight at 4°C. The signal intensity was quantified by
densitometry using a software (SynGene, Frederick, MD, U.S.A.).

**Immunohistochemistry:**
Immunostaining was performed as described previously (26). Briefly, the eyes were cross sectioned vertically through the center of the cornea and optic nerve, and both halves of the eyeball were embedded with the center facing down. Serial cryosection of 5 µm thickness was blocked with 10% goat serum in 0.1% Triton X-100 and 3% BSA in PBS. Following PBS washes, the antibody specific for the His-tag was added and incubated with the sections at 4°C overnight. The sections were rinsed several times with PBS and incubated with an FITC conjugated anti-mouse IgG antibody for 1 h. The slides were then rinsed in cold PBS and viewed under a fluorescence microscope.

**Real-time reverse transcription-polymerase chain reaction (RT-PCR):** The reverse transcription and real-time PCR were performed as described previously (26). The primers used for human VEGF (5' CAGACGGAGAAAGCATTTG and 3' TGGTCTCCGAAACCCTGAGG) amplified a 180-bp fragment of VEGF. The 18S rRNA was amplified using primers (5' TGCTGCAGTTAAAACTCGT, and 3' GGCTGTGCTTTGAACACTTA) for normalization of the VEGF mRNA levels.

**Rat models of OIR and STZ-induced diabetes:** All the animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Brown Norway (BN) rats (Charles River Laboratories) were employed for the OIR model following an established protocol (27). OIR rats received an intravitreal injection of K5-NP into the right eyes and the same dose of Control-NP into the left eyes at age of P12. Diabetes was induced and monitored in adult BN rats by an intraperitoneal injection of STZ (50 mg/kg in 10 mM of citrate buffer, pH 4.5), as described previously (14; 28).

Diabetic rats at 2 weeks after the onset of diabetes received an intravitreal injection of Control-NP and K5-NP in the treatment group and the same dose of Control-NP in the control group.

**Retinal vascular permeability assay:** Retinal vascular permeability was measured using the Evans blue-albumin leakage assay following an established protocol (29). Concentrations of Evans blue in the retina were normalized by total retinal protein concentrations and by Evans blue concentrations in the plasma.

**Fluorescein retinal angiography and quantifying pre-retinal vascular cells:** Retinal vasculature was visualized by fluorescein angiography as described (13; 27). The neovascular area was measured in the retina using SPOT software (Diagnostic Instruments, Sterling Heights, MI). For quantification of pre-retinal vascular cells, eyes were fixed, sectioned and stained as described previously (27). The pre-retinal nuclei were counted, averaged and compared using Student’s t test.

**Electroretinogram (ERG) recording:** Rats were dark adapted for at least 12 h. The rats were anesthetized, and pupils dilated with topical application of 2.5% phenylephrine and 1% tropicamide. ERG responses were recorded with a silver chloride needle electrode placed on the surface of the cornea after 1% tetracaine topical anesthesia. A reference electrode was positioned at the nasal fornix, and a ground electrode on the foot. The duration of light stimulation was 10 mSec. The band pass was set at 0.3 to 500 Hz. Fourteen responses were recorded and averaged, with flash intervals of 20 sec. For quantitative analysis, the B-wave amplitude was measured between A- and B-wave peaks. The ERG waveforms of both eyes in the same animal were simultaneously recorded and compared as the right-to-left–eye ratio of B wave amplitude.
Measurement of nuclear levels of hypoxia-inducible factor-1 (HIF-1): Nuclear and cytosolic fractions were isolated using the Fractionation System Kit (Biovision, Mountain View, CA). Nuclear and cytosolic protein concentrations were measured using the Bradford assay. The proteins were blotted with an antibody specific for HIF-1α (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:500 dilution.

RESULTS
Characteristics of K5-NP. Nanoparticle size, polydispersity index, zeta-potential, and plasmid loading were measured with four different nanoparticle batches, and various parameters are shown below (mean ± SD). The nanoparticle formulation exhibited a mean hydrodynamic diameter of 260 ± 30 nm, a polydispersity index of 0.28 ± 0.005, zeta-potential of 8.4 ± 2.4 mV, and a plasmid loading of 11 ± 1.4 µg/mg particles.

K5 expression from K5-NP in cultured cells. ARPE19 cells were transfected with 10 µg of K5-NP or Control-NP for 3 days. The concentrated conditioned serum-free medium from the transfected cells was blotted separately with the anti-K5 and anti-His-tag antibodies. Both of the antibodies detected significant amounts of K5 with the expected molecular weight secreted from the cells transfected with K5-NP, but not from the cells transfected with Control-NP (Fig. 1A).

The K5 expression was also determined by immunocytochemistry using the anti-His-tag antibody. The antibody detected K5 signal in the cells transfected with K5-NP, but not in the cells transfected with Control-NP (Fig. 1B, 1C).

Specific inhibition of endothelial cell growth and VEGF over-expression by K5-NP. To determine the biological effect of K5-NP, primary bovine retinal capillary endothelial cells (BRCEC) and ARPE19 cells were treated with K5-NP for 3 days, and viable cells were quantified using the MTT assay. As shown in Figures 2A and 2B, K5-NP induced a dose-dependent decrease in viable BRCEC numbers, when compared to the untreated cells and the cells treated with Control-NP (Fig. 2A). In contrast, the same K5-NP treatment did not result in significant reduction of viable cells in ARPE19 (Fig. 2B), suggesting that the inhibitory effect of K5-NP on cell growth is endothelial cell-specific.

Our previous studies showed that K5 inhibits angiogenesis via down-regulation of VEGF expression (30). Here we evaluated the effect of K5-NP on VEGF expression by measuring VEGF secretion and VEGF mRNA levels. As shown by ELISA, ARPE19 cells over-expressed VEGF after exposure to hypoxia (1% O₂). K5-NP treatment significantly reduced the VEGF secretion induced by hypoxia, to a level comparable to that from the cells under normoxia (Fig. 2C). Similarly, real-time RT-PCR showed that K5-NP significantly down-regulated the VEGF mRNA expression under hypoxia, while Control-NP did not affect VEGF expression under the same conditions (Fig. 2D).

The effects of K5-NP were also evaluated in cultured rMc-1, a cell line derived from rat retinal Müller cells (31). As shown by Western blot analysis, K5-NP attenuated the over-expression of VEGF and decreased HIF-1α levels in Müller cells cultured under hypoxia (1% O₂) (Fig. 2E).

Sustained expression of K5 in the retina after an intravitreal injection of K5-NP. To examine the expression of K5 from K5-NP in the retina, K5-NP was injected into the vitreous of the right eye (8.8 µg/eye) and the same amount of Control-NP into the left vitreous of 4 OIR rats at age of postnatal day 12 (P12), after exposure to 75% oxygen. At age of P18, the immunohistochemistry was performed on retinal sections using the anti-His-tag antibody. The immunostaining detected high levels of K5 expression in the inner retina in the eyes injected with K5-NP,
but not in the contralateral control eyes (Fig. 3A-D).

To determine the duration of the K5 expression after a single injection of K5-NP, the retinas were dissected and homogenized at 1, 2, 3 and 4 weeks after an intravitreal injection of K5-NP in adult rats (3 rats per time point) for Western blot analysis using the anti-His-tag antibody. K5 was detected with an expected molecular weight in the retinas injected with K5-NP at all the time points analyzed (Fig. 3E), but not in the retinas injected with the Control-NP.

**K5-NP reduces retinal vascular leakage in OIR rats.** To evaluate the effect of K5-NP on retinal vascular leakage, K5-NP was injected intravitreally into the right eyes (2.2 or 8.8 µg/eye) at age of P12 and the same amount of Control-NP into the contralateral eyes. Retinal vascular leakage was measured at P16 using the Evans blue-albumin leakage method and compared between the contralateral eyes. The results showed that the eyes injected with 8.8 µg of K5-NP had significantly lower vascular permeability than that in the contralateral retinas injected with Control-NP (P=0.011, n=7) (Fig. 4B). The injection of 2.2 µg of K5-NP did not result in a statistically significant decrease of vascular leakage (P=0.0531, n=7) (Fig. 4A).

**K5-NP inhibits ischemia-induced retinal neovascularization.** To evaluate the effect of K5-NP on retinal neovascularization, K5-NP was injected intravitreally into the right eyes (8.8 µg/eye) of the OIR rats at P12 and Control-NP into the left eyes. The retinal vasculature was visualized and examined by fluorescein retinal angiography at P18. Retinal angiographs on retinal flat mounts showed that the eyes injected with Control-NP developed severe retinal neovascularization in the OIR rats (Fig. 5A, 5C). In contrast, a single K5-NP injection ameliorated the retinal neovascularization in the same rats by decreasing neovascular areas and non-perfusion areas in the retina (Fig. 5B, 5D). The neovascularization was semi-quantified by measuring the ratio of the neovascular area to the total retina area, which showed that the eyes injected with K5-NP have significantly decreased retinal neovascular areas in the OIR rats, compared to those injected with Control-NP (Fig. 5E).

The effect of K5-NP on pre-retinal neovascularization was also quantified by counting vascular cells growing into the vitreous space (pre-retinal vascular cells) on 8 non-continuous cross-sections from each eye following an established method (27). The result showed that the eyes injected with K5-NP have significantly fewer pre-retinal neovascular cells, compared to the contralateral eyes injected with Control-NP (Fig. 6).

**K5-NP reduces retinal vascular leakage and blocks expression of VEGF and ICAM-1 in STZ-induced diabetic rats.** To determine the effect of K5-NP on retinal inflammation, K5-NP was injected intravitreally into STZ-induced diabetic rats, with the same dose of Control-NP as vehicle control. As VEGF and ICAM-1 are both known to play important roles in the retinal inflammation and BRB breakdown in diabetes, we measured retinal levels of ICAM-1 and VEGF from non-diabetic, STZ-induced diabetic, diabetic rats treated with Control-NP and K5-NP using Western blot analysis. Four weeks following the injection, the retinas from rats injected with K5-NP showed decreased retinal VEGF and ICAM-1 levels by more than 50%, compared to that in the retina injected with Control-NP and to that in untreated diabetic rats (Fig. 7A).

Consistently, leukostasis assay showed that K5-NP also decreased adherent leukocytes in the retinal vasculature of diabetic rats (Suppl. Fig. 1). Retinal vascular permeability in the retinas of non-diabetic and STZ-induced diabetic rats, and diabetic rats treated with Control-NP and K5-NP was measured using the Evans blue-albumin leakage method. As
shown by retinal vascular permeability assay, K5-NP reduced retinal vascular leakage by 30% in STZ-induced diabetic rats, 4 weeks after the injection of K5-NP (P<0.001, Fig. 7B), suggesting that the effect of K5-NP on retinal vascular leakage lasted for at least 4 weeks after a single injection. In contrast, Control-NP injection did not result in any significant difference in retinal levels of VEGF and ICAM-1 or retinal vascular leakage in the diabetic rats.

**Lack of detectable toxicities to the structure and function of the retina.** Possible toxicities of K5-NP were evaluated structurally by histological analysis. The eyes from animals with K5-NP injection and those with Control-NP injection were sectioned, stained and examined under light microscope at 1, 2 and 4 weeks after the injection. No difference was observed in number of retinal nuclear layers or thickness of the retina between the animals injected with K5-NP and the control (Fig. 8A-8F). We also measured the retinal thickness at the same six retinal locations in each eye as described in Supplemental Methods. There is no significant difference in retinal thickness in the rats treated with K5-NP and controls (Suppl. Fig. 2). Similarly, K5-NP did not increase apoptotic cells in the retina (Suppl. Fig. 3.).

Possible adverse effects of K5-NP on visual functions were evaluated by ERG recording in normal and diabetic rats. As shown in Figure 8, the amplitudes of A or B waves were not significantly different between the Control-NP or K5-NP treated rats and un-treated rats. STZ-induced diabetic rats showed ERG responses similar to the non-diabetic rats (Fig. 8G, 8H).

**K5-NP inhibits the activation of HIF-1.** To investigate the mechanism responsible for the anti-angiogenic activity of K5-NP, we have measured the effect of K5-NP on HIF-1 activation, as HIF-1 is the key transcription factor activating VEGF and is known to play a key role in retinal neovascularization (7). HIF-1α nuclear translocation is a critical step in the activation of HIF-1, and thus, we measured cytosolic and nuclear levels of HIF-1α in the human Müller cell line treated with K5-NP under hypoxia. As shown by Western blot analysis of isolated nuclear proteins, exposure of the cells to hypoxia (1% oxygen) increased HIF-1α levels in the nuclei (Fig. 9A). K5-NP, but not Control-NP, completely blocked the increase of nuclear HIF-1α levels under hypoxia (Fig. 9A).

Similarly, nuclear translocation of HIF-1α was also determined in the retina of the OIR rats by immunostaining of HIF-1α. OIR rats showed high levels of HIF-1α in the nuclei in the inner retina injected with Control-NP (Fig. 9B-D). In the contralateral eyes injected with K5-NP, the HIF-1α signal in the nuclei of retinal cells was apparently decreased (Fig. 9E-G), suggesting that K5-NP inhibited HIF-1α nuclear translocation.

**DISCUSSION**

Retinal vascular leakage, the direct cause of diabetic macular edema, and retinal neovascularization are the major causes of vision loss in diabetic patients (1). Long-term suppression of retinal vascular leakage and pathologic retinal neovascularization are the goals for the treatment of diabetic retinopathy. Although previous studies showed that peptide angiogenic factors such as K5, angiotatin, etc. effectively reduce vascular leakage and inhibit retinal neovascularization in animal models (14; 32), the difficulty in achieving sustained ocular delivery of the peptides represents a major hurdle for their therapeutic applications. The present study reports the first approach to combine an endogenous angiogenic inhibitor with nanotechnology in the treatment of diabetic retinopathy. Our results showed that an intravitreal injection of K5-NP mediated high-level and sustained K5 expression in the
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retina and resulted in sustained reductions of retinal vascular leakage in STZ-induced diabetic and OIR models. Further, K5-NP effectively blocked ischemia-induced retinal neovascularization. This study also identified a novel anti-inflammatory effect of K5 in the retina of diabetic rats. Moreover, the K5-NP injection did not result in any detectable toxicity to retinal structure and function. This study revealed therapeutic potential of nanoparticle-mediated gene delivery of angiogenic inhibitors in the treatment of diabetic retinopathy.

Nanoparticles formulated using PLGA polymers are recently being investigated as a new gene delivery system because of their sustained release characteristics, biocompatibility, biodegradability and ability to protect DNA from degradation in lysosomes (33; 34). Our immunoblotting and double immunostaining results showed that K5-NP mediated high-level and sustained K5 expression in cultured cells and in the inner retina, suggesting that K5-NP is efficiently internalized and released into the cytoplasmic compartment rather than being retained in the degradative lysosomal compartment. Moreover, the expressed K5 is secreted into the extracellular space and has biological activity of specifically inhibiting endothelial cell growth and VEGF over-expression under hypoxia and in the retina of STZ-induced diabetes.

Cytotoxicity is a potential concern in some nanoparticle-mediated gene deliveries. We have determined potential cytotoxicities of K5-NP in cultured ARP19 cells and in the retina. In ARPE19 cells treated with K5-NP, no significant reduction in cell viability was observed. In contrast, the same concentrations of K5-NP significantly inhibited endothelial cell growth, suggesting an endothelial cell-specific inhibitory effect of K5-NP. After an intravitreal injection of K5-NP, no significant alterations were observed in the retinal histology at any of the time points analyzed.

Moreover, analyses of the ERG response, retinal thickness and cell death in the retina suggest that K5-NP does not have any detectable side effects on retinal structure and functions. These data suggest that K5-NP has no severe toxicities at the dose used.

Our previous studies demonstrated that the anti-angiogenic activity of K5 is through down-regulating the expression of VEGF (30). As VEGF is a major permeability and angiogenic factor, down-regulation of VEGF expression is believed to be responsible for the effects of K5 on vascular leakage and retinal neovascularization (14; 15). To confirm whether the expressed K5 from nanoparticles plays a role in blocking the VEGF over-expression under hypoxia, we have measured VEGF expression in RPE cells and Müller cells. Exposure of the cells to hypoxia increased the VEGF expression. The over-expression of VEGF under hypoxia can be effectively blocked by K5-NP, suggesting that the mechanism underlying the vascular activities of K5-NP is identical to that of the K5 peptide.

The OIR model is commonly used for studies of proliferative diabetic retinopathy (35-37). Although it is not a diabetic model, the pathology in the retina in this model is similar to that in diabetic retinopathy in humans (27). Moreover, VEGF over-expression has been shown to be the key pathogenic factor for retinal vascular leakage and neovascularization in this model, a pathogenic mechanism similar to that in diabetic retinopathy (8; 38). Our results showed that K5-NP reduced retinal vascular permeability in a dose-dependent manner, demonstrating a beneficial effect on vascular leakage induced by hypoxia.

To evaluate the effect of K5-NP on retinal neovascularization in the OIR model, we used fluorescein angiography on flat-mounted retinas. The results showed that a single injection of K5-NP significantly reduced retinal neovascular area and non-
perfusion area which have been shown to correlate with the severity of diabetic retinopathy (27; 35). Since pre-retinal neovascularization, i.e., neovasculature growing into the vitreous space, is a characteristic of proliferate diabetic retinopathy, we have also evaluated the effect of K5-NP on pre-retinal neovascular events. The results showed that K5-NP significantly reduced pre-retinal neovascular cells, compared to the contralateral eyes injected with Control-NP. These results demonstrate that K5-NP attenuates ischemia-induced retinal neovascularization. A limitation of the OIR model, however, is that both the vascular leakage and retinal neovascularization are transient, with peaks at P16 and P18, respectively (27; 28).

Sustained expression is a goal of gene delivery. In the entire experimental period (4 weeks), K5-NP mediated efficient expression of K5 in the retina. To evaluate the sustained effects of K5, we have employed the STZ-diabetic rat model, a commonly used type-1 diabetic model. As STZ-induced diabetic rats are known to develop vascular leakage and retinal inflammation but not neovascularization, we measured the effect of K5-NP on retinal vascular leakage and inflammation in the STZ-induced diabetic model. Our results showed that K5-NP reduced retinal vascular leakage for at least 4 weeks after a single injection in diabetic rats. Further, K5-NP attenuated the over-expression of VEGF and ICAM-1, two major inflammatory factors (3; 39). Further, K5-NP also decreased leukostasis in the retina of the diabetic rats. These results indicate that K5-NP has a novel anti-inflammatory activity.

In conclusion, the present study demonstrates that PLGA nanoparticles can be used for gene delivery of angiogenic inhibitors into the retina without toxicity. The nanoparticle-mediated non-viral gene delivery of angiogenic inhibitors has therapeutic potential for the treatment of diabetic macular edema and retinal neovascularization associated with several ocular disorders such as diabetic retinopathy.

ACKNOWLEDGEMENTS

This study was supported by NIH grants EY015650, EY12231, EY019309 (J-x. Ma) and EY017045 (to UBK via Emory University), a grant P20RR024215 from the National Center for Research Resources and a grant from OCAST, research awards from ADA and JDRF.
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**FIGURE LEGENDS**

**Figure 1. K5 expression from K5-NP in vitro.** ARPE19 cells were grown to 70% confluence in a medium containing 5% FBS. The culture medium was replaced by a serum-free medium. Control-NP and K5-NP were separately added into the medium to 1 µg/mL (plasmid DNA concentration) and incubated with the cells for 72 h. (A) The medium was collected and concentrated. Total protein concentrations in the media were measured using the Bradford assay. The same amount of total proteins (20 µg) was applied for Western blot analysis separately using an antibody specific for human K5 and an anti-His-tag antibody. Lane 1, molecular weight markers; 2, medium from cells treated with K5-NP; 3, untreated cells; 4, treated with Control-NP; 5, purified K5 peptide as positive control. (B, C) ARPE19 cells were grown overnight on glass slides, treated with K5-NP and Control-NP at 1 µg/mL for 72 h, washed and fixed. The cells were immunostained with a monoclonal antibody against the His-tag using a 3, 3’-diaminobenzidine color reaction which shows brown color in cells with positive immunostaining. (B) 100×, (C) 400×.

**Figure 2. Inhibitory effect of K5-NP on endothelial cell growth and VEGF over-expression under hypoxia.** (A) BRCEC grown in media containing 1% FBS were treated with K5-NP at the concentrations as indicated for 72 h. The viable cells were quantified using the MTT assay. K5-NP induced a dose-dependent decrease of cell viability in BRCEC. (B) At the same concentrations, K5-NP did not inhibit ARPE19 cell growth. (C) ARPE19 cells were treated separately with 1 µg/mL Control-NP and K5-NP for 72 h. The culture medium was replaced with a serum-free medium, and the cells were exposed to hypoxia for 24 h. VEGF secreted into the medium was measured by ELISA using an ELISA kit (R&D Systems Inc., Minneapolis, MN) and normalized by total protein concentrations in the medium. (D) Total RNA was
extracted from the treated ARPE19 cells, and VEGF mRNA levels were quantified by real-time RT-PCR and normalized by 18S rRNA levels. Values are mean ± SD (n=3). (E) Müller cells were treated separately with 1 µg/mL Control-NP and K5-NP for 72 h. The culture medium was replaced with a serum-free medium, and the cells were exposed to hypoxia for 24 h. VEGF and HIF-1α levels were analyzed by immunoblotting and normalized by β-actin levels.

**Figure 3. K5 expression in the rat retina after an intravitreal injection of K5-NP.** (A-D) K5-NP was injected into the vitreous of the right eyes and Control-NP into the contralateral eyes of 4 OIR rats at age of P12. K5 expression was examined at age of P18 by immunohistochemistry in ocular sections using an anti-His-tag antibody (green). The nuclei were counter-stained with DAPI (red). (A), a representative immunostaining image from the eyes injected with K5-NP and (C), injected with Control-NP. (B) and (D) are phase contrast images of the same areas of (A) and (B), respectively. Scale bar: 20 µm. (E) K5-NP and Control-NP were separately injected into the vitreous. The retinas were dissected at 1, 2, 3 and 4 weeks after the injection of K5-NP or Control-NP (3 rats per time point). The same amount of proteins (100 µg) from each retina was loaded for Western blot analysis using the anti-His-tag antibody. The same membrane was stripped and re-blotted with an anti-β-actin antibody. No K5 expression was found in the retinas injected with Control-NP, whereas K5 expression was detected in all of the retinas with the K5-NP injection.

**Figure 4. Effect of K5-NP on retinal vascular leakage in OIR rats.** OIR rats received an intravitreal injection of 2.2 µg (A) or 8.8 µg (B) of K5-NP into the right eyes and the same dose of Control-NP into the left eyes at age of P12. Retinal vascular permeability was measured using the Evans blue-albumin leakage method at P16. The vascular leakage was normalized by total protein concentrations in the retina, averaged within the group (mean±SD, n=7), and compared between contralateral eyes using paired Student’s t test. The eyes injected with 8.8 µg of K5-NP showed a significantly lower retinal vascular permeability compared to the contralateral eye (P=0.011) (B).

**Figure 5. Effect of K5-NP on retinal neovascularization in OIR rats.** K5-NP was injected into the vitreous of the right eyes (8.8 µg/eye) and the same amount of Control-NP into the contralateral eyes of 7 OIR rats at age of P12. Retinal vasculature was examined using fluorescein angiography at P18 as described in Methods. (A & C) Representative retinal angiographs from the eyes injected with Control-NP; (B & D) representative angiographs from the K5-NP-injected eyes (40× in A & B; 100× in C & D). The white arrow in (C) indicates a neovascular area in the retina. Scale bar: A&B, 100 µm; C&D, 40 µm. (E) Retinal neovascularization was quantified by measuring the neovascular area in the retina and expressed as % of the total retina area (mean±SD, n=7). The difference of the neovascular area was compared between the contralateral eyes using paired Student’s t test.

**Figure 6. Effect of K5-NP on pre-retinal neovascularization in OIR rats.** K5-NP was injected intravitreally into the right eyes, while Control-NP into the left eyes of 6 OIR rats at age P12. The eyes were fixed, sectioned and stained with hematoxylin and eosin at P18. (A & B) Representative sections from the eyes injected with Control-NP (A) and from that with K5-NP (B) injection. Scale bar: 40 µm. (C) Pre-retinal vascular cells were counted in 8 non-continuous sections per eye and averaged as described in Methods. The average numbers of pre-retinal
vascular cells (mean±SD, n=6) were compared between the eyes injected with K5-NP and those with Control-NP using paired Student’s t test.

**Figure 7. Effect of K5-NP on retinal vascular leakage and expression of VEGF and ICAM-1 in diabetic rats.** STZ-induced diabetic rats at 2 weeks after the onset of diabetes received an intravitreal injection of 5 µg/eye of K5-NP in the treatment group and the same dose of Control-NP in the control group. (A) Four weeks after the injection, retinal levels of ICAM-1 and VEGF from non-diabetic control, un-treated diabetic rats, diabetic rats treated with Control-NP and K5-NP were determined using immunoblotting. The same membrane was stripped and re-blotted with the anti-His-tag and anti-β-actin antibodies. Retinal levels of VEGF and ICAM-1 in diabetic rats treated with Control-NP and K5-NP were quantified by densitometry and expressed as % of that in non-diabetic rat retinas (mean ± SD, n=3). (B) Retinal vascular permeability in the retina of non-diabetic rats, un-treated diabetic rats, diabetic rats treated with Control-NP and K5-NP was measured using the Evans blue-albumin leakage method at 4 weeks after the injection of Control-NP or K5-NP, normalized by the total protein concentration in the retina and the Evans blue concentration in the blood and expressed as ng dye per mg of retinal proteins (mean ± SD, n=8).

**Figure 8. Histology and ERG response in the eyes injected with K5-NP.** Adult rats received an intravitreal injection of K5-NP or Control-NP. The animals were euthanized, and the eye sections were stained with H&E (3 rats per time point). (A-F) representative images of the eyes at 1 (A, D), 2 (B, E) and 4 (C, F) weeks after the injection. Scale bar: 20 µm. (G, H) ERG was recorded from 6 rats at 4 weeks after the injection of K5-NP and Control-NP to non-diabetic and diabetic rats, and age-matched untreated non-diabetic and diabetic rats. Amplitudes of A and B waves from scotopic and photopic ERG were averaged and compared (mean±SD, n=6).

**Figure 9. Blockade of nuclear translocation of HIF-1α by K5-NP.** (A) BRCEC were treated with K5-NP or Control-NP for 48 h under normoxia or hypoxia (1% oxygen). The cytosol and nucleus were isolated, and the same amount of proteins (30 µg) from each sample was used for Western blot analysis using an anti-HIF-1α antibody. The membranes were stripped and re-blotted with antibodies for β-actin and nuclear protein TBP. Cytosolic and nuclear levels of HIF-1α were semi-quantified by densitometry, normalized by β-actin and TBP, respectively, and expressed as % of non-diabetic control (mean±SD, n=3). (B-G) Three OIR rats received an intravitreal injection of Control-NP in the left eye (B-D) and K5-NP into the right eye (E-G) at P12. The eyes were fixed and sectioned at P16. The retinal sections were stained with the antibody specific for HIF-1α (green), and the nuclei counterstained with DAPI (red). (B & E) HIF-1α immunostaining; (C & F) DAPI staining of the nuclei; (D & G) merged images of HIF-1α and DAPI staining. Note that the nuclei with increased HIF-1α signal superimposed on DAPI staining show orange color in (D). White arrows indicate different intensities of HIF-1α staining in the nuclei of inner retinal cells in (D & G). Scale bar: 10 µm.
Figure 1.
Figure 2.
Figure 3.
**Figure 4**

![Graph A](image)

**Figure 5.**

![Images A to D](image)

![Graph E](image)
Figure 6

A
Control -NP

B
K5 -NP

C

![Graph showing comparison between Control-NP and K5-NP](image)

P<0.01
Figure 7

A

Anti-ICAM-1
Anti-VEGF
Anti-His-tag
Anti-β-actin

B

ICAM-1 Level (% of Non-diabetic)

VEGF Level (% of Non-diabetic)

Permeability (μg/mg)

P < 0.001

P < 0.001

P > 0.5

P > 0.5

P < 0.01

P < 0.01

P < 0.01

Normal
STZ
Control-NP
STZ
K5-NP
Normal
STZ
Control-NP
STZ
K5-NP
Normal
STZ
Control-NP
STZ
K5-NP
Figure 8

Nanoparticles for diabetic retinopathy
Figure 9.
