Anti-inflammatory and Antiangiogenic Effects of Nanoparticle-Mediated Delivery of a Natural Angiogenic Inhibitor

Ji Jin,^{1,2} Kevin K. Zhou,² Kyoungmin Park,² Yang Hu,² Xun Xu,³ Zhi Zheng,^{*,3} Puneet Tyagi,⁴ Uday B. Kompella,⁴ and Jian-xing Ma^{*,2}

PURPOSE. The purpose of this study was to evaluate the inhibitory effects of the nanoparticle-mediated delivery of plasminogen kringle 5 (K5) on choroidal neovascularization (CNV) and retinal inflammation.

METHODS. CNV was induced by laser in adult rats. Nanoparticles with an expression plasmid of K5 (K5-NP) were injected into the vitreous. K5 expression was detected by immunohistochemistry. The CNV area was measured after fluorescein angiography. Retinal vascular permeability was quantified with Evans blue as a tracer. Expression of vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)- α , monocyte chemoattractant protein (MCP)-1, and intercellular adhesion molecule (ICAM)-1 was measured by Western blot analysis or ELISA and real-time RT-PCR.

RESULTS. Intense K5 expression was detected in the retina 2 weeks after the injection of K5-NP. Areas of CNV were significantly decreased in the K5-NP treatment group compared with that in the control-NP group. The K5-NP injection also significantly reduced vascular permeability. The expression of VEGF was downregulated by K5-NP at both the protein and mRNA levels. Moreover, K5-NP also inhibited expression of TNF- α and ICAM-1. Similarly, K5-NP decreased retinal levels of total β -catenin. In cultured cells, K5-NP suppressed hypoxia-induced secretion of MCP-1 and TNF- α .

CONCLUSIONS. K5 has a novel anti-inflammatory activity. K5-NP mediates a sustained inhibitory effect on CNV and thus has therapeutic potential for age-related macular degeneration. (*In-*

vest Ophthalmol Vis Sci. 2011;52:6230–6237) DOI:10.1167/iops.10-6229

Choroidal neovascularization (CNV) refers to abnormal vessel growth of the choroidal capillaries under the retinal pigment epithelium (RPE) and through Bruch's membrane into the subretinal space. The abnormal vessels in the subretinal space can lead to hemorrhage, scar formation, and exudation, resulting in retinal detachment and vision loss.^{1,2} CNV occurs in patients with the wet form of age-related macular degeneration (AMD), the most common cause of blindness among the elderly in developed countries.^{3,4} A strategy for the treatment of AMD includes prevention of the development and progression of CNV.

The therapies for CNV can be categorized into either destructive or pathway-based therapies.⁵ Destructive therapies include laser photocoagulation and photodynamic procedures. Although the destructive therapies do deliver some beneficial effects in reducing the severity of CNV, their therapeutic application is limited by the patient's history and common complications. One potential complication is scar formation caused by the laser, which can reduce central vision. Pathway-based therapies are aimed mainly at inhibiting the process of angiogenesis by either blocking a proangiogenic factor or stimulating an antiangiogenic factor.⁶ Recently, antivascular endothelial growth factor (VEGF) compounds have shown encouraging efficacy in treating wet AMD.⁷ However, the current anti-VEGF molecules are associated with short duration of the efficacy and require repetitive intravitreal injections.⁷ Therapies which can achieve long-term suppression of CNV remain to be developed.

Plasminogen kringle 5 (K5) is an 80-amino-acid proteolytic fragment of plasminogen.⁸ Previously, K5 has displayed potent antiangiogenic activity in neovascularization models.^{8,9} It has been shown that K5 directly targets endothelial cells and tumor cells and induces apoptosis.^{10,11} This effect has been suggested to be through interactions with glucose-regulated protein 78 (GRP78) on the cell surface.¹¹ Furthermore, K5 has been shown to inhibit VEGF expression through the HIF-1 pathway.¹² Our previous study showed that K5, when injected intravitreally, effectively inhibits retinal NV in oxygen-induced retinopathy (OIR).¹³ Injection of the K5 peptide also reduces retinal vessel leakage in both of the OIR and streptozotocin (STZ)-induced diabetes models.¹⁴ However, these effects are transient, possibly due to the short half-life of the K5 peptide in the vitreous and retina.

Nonviral gene delivery systems are being increasingly advocated in gene therapy because of their low immunogenicity, unlimited payload capacity, absence of endogenous virus recombination and low production costs.¹⁵ PLGA or poly(lactide-co-glycolide acid), a biocompatible and biodegradable polyester co-polymer of PLA and PGA, has been recognized for its

From the ¹Department of Ophthalmology, The Children's Hospital, Zhejiang University School of Medicine, Hangzhou, China; the ²Department of Physiology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; ³The First People's Hospital, Shanghai Jiaotong University, Shanghai, China; and the ⁴Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Aurora, Colorado.

Supported by National Institutes of Health (NIH) Grants EY012231, EY018659, EY019309, and P20RR024215 from the National Center for Research Resources, grants from the American Diabetes Association, and Grant P20RR024215 to the Diabetic Animal Core Facility from the Centers of Biomedical Research Excellence (COBRE), NIH.

Submitted for publication July 16, 2010; revised November 16 and December 13, 2010; accepted December 16, 2010.

Disclosure: J. Jin, None; K.K. Zhou, None; K. Park, None; Y. Hu, None; X. Xu, None; Z. Zheng, None; P. Tyagi, None; U.B. Kompella, None; J. Ma, None

*Each of the following is a corresponding author: Jian-xing Ma, 941 Stanton L. Young Boulevard, BSEB 328B, Oklahoma City, OK 73104; jian-xing-ma@ouhsc.edu.

Zhi Zheng, Department of Ophthalmology, First People's Hospital of Shanghai, Shanghai Jiaotong University, Shanghai 200080, China; zzheng8@yahoo.com.cn.

ability to deliver genes.¹⁵ Chitosan-modified PLGA nanoparticles have greater potential as gene carriers.¹⁶ PLGA nanoparticle is considered a promising nonviral vehicle for gene delivery, due to its large capacity, biocompatibility, and biodegradable feature. In a recent study, we have shown that PLGA nanoparticles containing an expression plasmid of K5 (K5-NP) mediated efficient and sustained expression of K5 in the retina. In addition, the K5-NP ameliorated retinal vascular leakage in the STZ-induced diabetic retinopathy model and attenuated retinal NV in the OIR model.¹⁷

Laser-induced CNV is widely used as a model for wet AMD, as it replicates its pathologic features, including inflammation and CNV. In the present study, we used the laser-induced CNV rat model to evaluate the sustained efficacy of K5-NP on CNV and explored its anti-inflammatory effect.

METHODS

Animals

All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the University of Oklahoma Institutional Animal Care and Use Committee on Animal Research. Male Brown Norway (BN) rats (Charles River Laboratories, Wilmington, MA) 8 weeks of age were divided into two groups (K5-NP and control-NP treatment groups) and anesthetized with 0.2 to 0.3 mL of a 1:1 mixture of ketamine (100 mg/mL) and xylazine (20 mg/mL) through intraperitoneal injection. Pupils were dilated with a topical application of 1% cyclopentolate and 1% atropine (Alcon Laboratories, Inc., Fort Worth, TX).

Induction of CNV

A diode red laser (810-nm wavelength; Keeler, Windsor, UK, and Oculight SLX; Iris Medical, Mountain View, CA) was used under a Fison indirect ophthalmoscope to induce CNV by rupturing Bruch's membrane in anesthetized rats. Four laser lesions were placed at four quadrants of each eye at equal distance from the optic disc. The visible spot size of the laser lesion was kept relatively constant. Laser parameter settings were 100 ms of exposure time and 275 mW of power. Only the eyes with visible vapor bubble formation at the laser lesion sites were included in the study. This laser procedure and the examination were performed by an experienced ophthalmologist. Laser spots with hemorrhage were excluded from further analysis. CNV was monitored with funduscopy and analyzed by fluorescein angiography of the RPE-choroid flat mount.

Preparation of the PLGA:Chitosan (CHN)-K5 Nanoparticles (K5-NP)

An expression plasmid of K5 was constructed as previously described.¹⁷ PLGA:Chitosan nanoparticles containing a K5 expression plasmid were prepared by using a previously reported emulsion-diffusion-evaporation technique with some modifications.¹⁸ Briefly, the PVA solution (1%, wt/vol) was prepared and then chitosan chloride (2.5 mg PCL 113; NovaMatrix, Philadelphia PA) was dissolved with stirring. The plasmid (0.35 mg/mL) in 10% (wt/vol) sodium sulfate was added to the chitosan solution with stirring. The solutions were then emulsified with a probe sonicator for 4 minutes. To the emulsion, approximately 30 mL of ultrapure water (Milli-Q H₂O; Millipore, Billerica, MA) was added and stirred on a magnetic plate stirrer to evaporate the solvent. The particle suspension was ultracentrifuged and resuspended, and the procedure was repeated twice. On final resuspension in the ultrapure water, the nanoparticle suspension was lyophilized to obtain dry particles.

Estimation of Plasmid Loading in Nanoparticles

For plasmid loading estimation, the lyophilized polymer (0.2 mg) was dispersed in 1 mL of methylene chloride to dissolve the polymer,

followed by extraction of the plasmid in 2 mL of Tris-EDTA (TE) buffer. An aliquot of the TE buffer fraction was analyzed at an absorbance of 260 nm, to determine the plasmid content per milligram of nanoparticles.

Intravitreal Administration of K5

Rats were randomly assigned to two injection groups: the K5-NP group and the control-NP (plasmid without K5 insert) group. The intravitreal drug delivery of the K5-loaded PLGA nanoparticles (200 μ L) was performed on the same day as the laser photocoagulation, according to a previously described method.¹⁷ Briefly, intravitreal injections of 10 μ g/ μ L K5-NP or control-NP were administered through a 32-gauge needle (Hamilton Co., Reno, NV) under a dissecting microscope immediately after laser lesioning. Intravitreal drug delivery was confirmed by observing the white K5-loaded PLGA nanoparticle suspension in the posterior vitreous under the microscope. Eyes with lens injury from the injection were excluded from the study.

Permeability Assay

Fourteen days after injection of K5-NP, retinal vascular permeability was determined by the Evans blue-albumin leakage assay in an established protocol.¹⁹ Briefly, Evans blue was injected intravenously and allowed to circulate for 2 hours. Evans blue has high affinity with serum albumin and forms Evans blue-albumin complex in the circulation. The animal was then perfused to remove Evans blue-albumin from the vasculature. The retina was dissected and homogenized. Evans blue was extracted, and its concentration measured and normalized by total retinal protein concentration.

Fluorescein Angiography and RPE-choroid Flat Mounts

CNV lesions were visualized by fluorescein angiography with high-molecular-weight fluorescein-dextran, as described.^{13,20} The RPE-choroid complexes (20 rats per group) were dissected and flat mounted on slides with glycerol-gelatin. Images were captured and evaluated under a confocal microscope with both a reflected fluorescence system for CKX 41 and a reference micrometer. The largest area of CNV was measured with image-analysis software (SPOT; Diagnostic Instruments, Sterling Heights, MI). The mean area of CNV was derived from measurements of all the CNV lesions (80 lesions per group) by a masked specialist.

Histologic and Immunohistochemical Analysis of CNV

The eyes (five rats per group) were enucleated, fixed, and sectioned. After they were washed in phosphate-buffered saline (PBS; pH 7.4), the sections were blocked with 5% horse serum for 1 hour and incubated with the primary antibody (goat anti-intercellular adhesion molecule-1 (ICAM-1), rabbit anti- β -catenin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-tumor necrosis factor (TNF)- α (1:500; Abcam, Cambridge, MA), or chicken anti-K5 antibodies overnight.¹⁷ Slides were rinsed with PBS and incubated with fluorescent dye-conjugated secondary antibodies (1:500; Alexa 488 and Alexa 546; Invitrogen, Carlsbad, CA; 1:400; anti-chicken [NL-557] and anti-goat [FITC]; R&D Systems Inc., MN). Specificity of the immunostaining was assessed by substitution of the primary antibody with the preimmune serum. Slides were rinsed with PBS and mounted with mounting medium containing DAPI (Vectashield; Vector Laboratories, Inc, Burlingame, CA). Images were captured and evaluated under a microscope with both a reflected fluorescence system for CKX 41 and a reference micrometer (Olympus, Tokyo, Japan).

Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA was prepared from rat (five rats per group) eye cups (RPE-choroid complex) individually (Trizol; Invitrogen, Carlsbad, CA)

FIGURE 1. K5 expression in the rat retina after intravitreal injection of K5-NP. K5-NP or control-NP were intravitreally injected into eyes with laser lesions. Two weeks after injection, K5 expression was examined by immunohistochemistry in ocular sections using an antibody specific for human K5 (red). The nuclei were counterstained with DAPI (blue). (A) A representative image from an eye injected with control-NP; (C) a representative image from an eye injected with K5-NP; (E) K5 immunostaining in a laser lesion area in the K5-NP treated group. (B, D, F) are the same areas of (A, C, E), respectively, after the DAPI staining was merged with K5 signals. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

and reverse transcribed with a cDNA synthesis kit (RevertAid First-Strand cDNA Synthesis; Fermentas, Burlington, ON, Canada). Real-time PCR was conducted with the SDS system (ABI 7000; Applied Biosystems, Foster City, CA) with SYBR green (TaKaRa Bio, Otsu, Shiga, Japan). Each PCR was performed in duplicate. Primers for human VEGF (5'-CAGAGCGGAGAAAGCATTTG-3' and 5'-TGGTTCGCCGAAACCCCTGAGG-3'), rat TNF- α (5'-AGCACAGAAAGCAT GATCCG-3' and 5'-CTGGGAGTGTGAGTCTAGTA-3') and rat ICAM-1 (5'-TCTCAGCAGACTCTTACATC-3' and 5'-GGTCTCCTTCTAGTCCTATA-3') amplified 180-bp fragments. The 18S rRNA was amplified with primers (5'-TGCTGCAGTTAAA AAGCTCGT-3', and 5'-GGCCTGCTTGAACA CTCTAA-3') for normalization of the VEGF, TNF- α , and ICAM-1 mRNA levels.

Western Blot Analysis

Eye cup homogenates (five rats per group) were prepared, and protein concentrations measured by the Bradford assay (Bio-Rad Laboratories, Munich, Germany). Equal amounts of proteins were resolved by 12% or 8% SDS polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked in a solution of 5% (wt/vol) nonfat milk powder in TBST for 1 hour and then blotted overnight at 4°C with antibodies for β -catenin, VEGF, ICAM-1 (1:500; Santa Cruz Biotechnology), or TNF- α (1:500; Abcam). After washes with TBST, the respective secondary antibodies were incubated with the membrane for 1 hour and visualized by enhanced chemiluminescence. For sequential blotting with additional antibodies, the membranes were stripped with a stripping buffer and reblotted with the indicated antibodies. Protein levels were semiquantified by densitometry and normalized to the β -actin levels.

In Vitro Assays

ARPE19 cells were treated with 1 μ g/mL control-NP or K5-NP for 72 hours. The culture medium was then replaced with serum-free medium, and the cells were exposed to hypoxia (1% oxygen) for 24 hours. Monocyte chemoattractant protein (MCP)-1 and TNF- α secreted into the medium were measured by ELISA (R&D Systems, Inc.) and normalized by total protein concentration in the conditioned medium.

Statistical Analysis

All results were expressed as the mean $\pm$ SD. Statistical analyses were performed with Student's *t*-test.

RESULTS

Efficient K5 Expression in the Neurosensory Retina after an Intravitreal Injection of K5-NP

An equal amount of K5-NP or control-NP was injected into the vitreous cavity of rats immediately after laser coagulation. Two weeks after injection, K5 expression in the retina was examined by immunohistochemistry with an antibody specific for human K5. Intensive K5 signal was detected primarily in the inner retina in the eyes injected with K5-NP, but not in those injected with control-NP (Figs. 1A–D). In the laser lesion areas of the retina in the K5-NP-treated group, significant K5 expression was detected in the neurosensory retina (Figs. 1E, 1F).

Suppression of Laser-Induced CNV by K5-NP

To determine whether K5-NP can attenuate laser-induced CNV, the CNV lesions in K5-NP or control-NP-injected RPE-choroid flat mounts were visualized by fluorescein angiography. Areas of CNV lesions were measured and averaged within each group

FIGURE 2. Suppression of laser-induced CNV by K5-NP. Two weeks after injection of control-NP (A) and K5-NP (B) into the vitreous of an eye with laser lesions, CNV was visualized by fluorescein angiography with FITC-dextran. (A, B) Representative micrographs of CNV lesions in an RPE-choroid flat mount in the control-NP treatment group (A) and K5-NP treatment group (B). (C) The CNV areas were measured and compared (mean $\pm$ SD, *n* = 20).

and compared. The areas of CNV in the K5-NP treatment group were decreased by 40%, compared with those with control-NP treatment ($P < 0.01$, $n = 20$; Fig. 2), suggesting that K5-NP suppresses laser-induced CNV.

K5-NP Reduced Retinal Vascular Leakage and VEGF Overexpression in the Laser-Induced CNV Model

To examine the prevalence of retinal vascular leakage after K5-NP injection, we measured retinal vascular permeability in the K5-NP- or control-NP-injected eyes with the Evans blue albumin leakage method. The eyes in the K5-NP treatment group showed significantly lower vascular permeability in the retina, compared with that in the control-NP treatment eyes, suggesting that K5-NP decreased retinal vascular leakage 14 days after injection (Fig. 3A).

As VEGF is a potent angiogenic and permeability factor, we also measured its expression levels in the eye. Real-time RT-PCR showed that VEGF mRNA levels in the eyes were significantly decreased by the K5-NP treatment, compared with that in the control-NP group (Fig. 3B). Furthermore, Western blot analysis showed that VEGF protein levels were significantly lower in the eyes in the K5-NP treatment group than in those of the control-NP group (Figs. 3C, 3D). Taken together, these results indicate that K5-NP suppressed angiogenesis and permeability in the CNV model.

K5-NP Downregulated Inflammatory Factors in the Laser-Induced CNV Eye

To determine whether K5 inhibited inflammation in our laser-induced CNV model, we measured the effect of K5-NP on

overexpression of TNF- α , a major inflammatory cytokine. As shown by Western blot analysis, levels of TNF- α in the K5-NP-injected eyes were significantly lower than in the control-NP CNV group (Figs. 4A, 4B). Real-time RT-PCR showed that TNF- α mRNA levels were also significantly decreased in the K5-NP CNV eyes (Fig. 4C), suggesting that K5-NP downregulates TNF- α expression. Immunohistochemistry showed that TNF- α was overexpressed primarily in the retina and RPE in the rats with laser-induced CNV. However, reduced intensities of the TNF- α immunosignals were observed in the K5-NP-injected eyes (Figs. 4D–G).

ICAM-1 is an adhesion molecule and is a major mediator of leukostasis in inflammatory responses. As shown by Western blot analysis, levels of ICAM-1 in the K5-NP CNV eyes were significantly decreased, compared with those in the control-NP CNV group (Figs. 5A, B). Furthermore, K5-NP treatment significantly suppressed mRNA expression of ICAM-1, as shown by real-time RT-PCR (Fig. 5C). Expression of ICAM-1 was observed primarily in the RPE layer of rats with laser-induced CNV. On K5-NP treatment, the levels of ICAM-1 were significantly decreased, compared with those in the control-NP CNV group (Figs. 5D–G). The decreased levels of the inflammatory factors TNF- α and ICAM-1 in the K5-NP CNV eyes indicate that K5-NP inhibited inflammation in this CNV model.

Attenuation of β -Catenin Accumulation in the K5-NP-Injected CNV Retina

β -Catenin is an essential effector of the Wnt/ β -catenin pathway and a regulator of inflammation and angiogenesis. To examine whether the K5-NP can regulate β -catenin levels in the CNV retina, we used Western blot analysis. Levels of

FIGURE 3. K5-NP reduced retinal vascular permeability and VEGF levels in eyes with laser-induced CNV. K5-NP and control-NP were separately injected into eyes with laser lesions. (A) Two weeks after injection, vascular permeability in the retina was measured with Evans blue as a tracer. The results were normalized by total retinal protein concentrations and expressed as micrograms of dye per milligram of protein in the retina (mean $\pm$ SD, $n = 5$). (B) VEGF mRNA levels in the eyes were measured with real-time RT-PCR and expressed as a percentage of that in the control-NP group (mean $\pm$ SD, $n = 5$). (C) The same amount of protein (50 μ g) from each eye was examined by Western blot analysis with the anti-VEGF antibody. The same membrane was stripped and reblotted with an anti- β -actin antibody. Each lane represents an individual rat. (D) VEGF levels were semiquantified by densitometry, normalized by β -actin levels, and expressed as a percentage of that in the control-NP group (mean $\pm$ SD, $n = 5$).

FIGURE 4. K5-NP downregulated TNF- α expression in the eyes with laser-induced CNV. K5-NP or control-NP were injected into the vitreous of rats with laser-induced CNV. The eyes were dissected 14 days after injection for the following analyses. **(A)** The same amount of eye cup proteins (50 μ g) from each rat was examined by Western blot analysis with the anti-TNF- α antibody. The same membrane was stripped and reblotted with an anti- β -actin antibody. Each lane represents an individual rat. **(B)** TNF- α levels were semiquantified by densitometry, normalized by β -actin levels, and expressed as a percentage of that in the control-NP group (mean $\pm$ SD, $n = 5$). **(C)** TNF- α mRNA levels in the eyes were measured with real-time RT-PCR, normalized by 18s RNA levels, and expressed as a percentage of that in the control-NP group (mean $\pm$ SD, $n = 5$). **(D–G)** TNF- α expression was examined by immunohistochemistry in ocular sections with an anti-TNF- α antibody (green). The nuclei were counterstained with DAPI (blue). **(D)** A representative immunostained image from the eyes in the control-NP group; **(F)** A representative immunostained image from the eyes in K5-NP group; **(E, G)** Immunostaining in **(D, F)** merged with DAPI staining.

β -catenin in the K5-NP CNV eyes were significantly lower than those in the control-NP CNV eyes (Figs. 6A, 6B). Similarly, retinal levels of NF- κ B, another transcription factor regulating inflammation, were reduced by K5-NP in the laser-induced CNV model (Figs. 6C, 6D).

Direct Inhibition of Inflammatory Cytokine Expression by K5-NP in Cultured RPE Cells

To confirm that K5-NP directly modulates the expression of inflammatory factors induced by hypoxia, we evaluated the effect of K5-NP on the hypoxia-induced overexpression of TNF- α and MCP-1 in cultured ARPE19 cells. Cells cultured under hypoxic or normoxic conditions were treated with either control-NP or K5-NP. As measured by ELISA, hypoxia significantly increased the secretion of MCP-1 and TNF- α . However, K5-NP treatment blocked the hypoxia-induced increase in MCP-1 and TNF- α secretion (Fig. 7), confirming the anti-inflammatory effect of K5-NP.

DISCUSSION

K5 is a proteolytic fragment of plasminogen and has been shown to have potent antiangiogenic properties in several angiogenic models.^{8,13} However, a major challenge in its therapeutic application is its short half-life in vivo. The present study demonstrated that nanoparticle-mediated gene delivery of K5 achieved sustained expression of K5 and

suppressed laser-induced CNV. Furthermore, K5-NP downregulated the expression of multiple inflammatory factors, establishing a newly identified anti-inflammatory activity of this natural antiangiogenic peptide.

Effective and long-term treatment of CNV in AMD remains one of the greatest challenges in clinical ophthalmology. The need for a successful treatment is augmented because of an increasing population over the age of 65. Previous studies showed that K5 is an endogenous angiogenic inhibitor that inhibits ischemia-induced retinal NV and vascular leakage in a diabetic model.^{13,14} However, the effects lasted only 2 days after an intravitreal injection of purified K5 peptide.²¹ Since NV is a chronic process in most ocular diseases, including AMD and DR, long-term efficacy of K5 is desirable. Recently, we have developed a PLGA-based K5-NP which mediates K5 expression in the retina for at least 4 weeks after a single intravitreal injection.¹⁷ In the present study, we applied K5-NP in the laser-induced CNV model. The results showed that K5-NP decreased CNV areas 2 weeks after injection, suggesting sustained antiangiogenic activity. Moreover, K5-NP reduced retinal vascular leakage for at least 2 weeks in the CNV model. These results suggest that the nanoparticle-mediated expression of natural antiangiogenic factors has great therapeutic potential for neovascular disorders.

Immunohistochemical analysis showed that intravitreal injection of K5-NP mediated K5 expression primarily in the inner

FIGURE 5. K5-NP downregulated ICAM-1 expression in the eyes with laser-induced CNV. K5-NP or control-NP were injected into the vitreous of laser-induced CNV rats. The eyes were dissected 14 days after injection. (A) The same amount of eye cup proteins (50 μ g) from each rat was examined by Western blot analysis with an anti-ICAM-1 antibody. The same membrane was stripped and reblotted with the anti- β -actin antibody. Each lane represents an individual rat. (B) ICAM-1 levels were semiquantified by densitometry, normalized by β -actin levels, and expressed as a percentage of that in the control-NP group (mean $\pm$ SD, $n = 5$). (C) ICAM-1 mRNA levels in the eyes were measured with real-time RT-PCR, normalized by 18s RNA levels, and expressed as a percentage of that in the control-NP group (mean $\pm$ SD, $n = 5$). (D–G) ICAM-1 expression was examined by immunohistochemistry in ocular sections using an anti-ICAM-1 antibody (green). The nuclei were counterstained with DAPI (blue). (D) A representative immunostained image from control-NP-injected eyes; (F) a representative immunostained image from K5-NP-injected eyes. (E, G) Immunostaining in (D, F) merged with DAPI staining.

retina, consistent with our previous studies in the diabetic model.¹⁷ This result suggests that K5-NP after intravitreal injection does not penetrate the outer retina. However, in the laser lesioned area, K5 expression was detected throughout all the layers of the retina. The high-level K5 expression in the outer retina in the lesioned area may explain its antiangiogenic

effect on the laser-induced CNV that occurs in the subretinal space.

VEGF is a potent angiogenic stimulator that promotes proliferation and migration of vascular endothelial cells and enhances vascular permeability.²² Furthermore, it plays a key role in pathologic NV in both AMD and DR.²³ Previous

FIGURE 6. K5-NP decreased β -catenin and NF- κ B levels in eyes with laser-induced CNV. K5-NP or control-NP were injected into the vitreous of laser-induced CNV rats. The eyes were dissected 14 days after injection. The same amount of eye cup proteins (50 μ g) from each rat was examined by Western blot analysis with an anti- β -catenin antibody (A) and anti-NF- κ B antibody (C). Each lane represents an individual rat. The same membrane was stripped and reblotted with an anti- β -actin antibody. (B) β -Catenin and (D) NF- κ B levels were semiquantified by densitometry, normalized by β -actin levels, and expressed as a percentage of that in control-NP group (mean $\pm$ SD, $n = 5$).

FIGURE 7. Direct effects of K5-NP on the expression of inflammatory cytokines in RPE cells. The direct effect of K5-NP on the expression of inflammatory cytokines was evaluated in cultured RPE cells. ARPE19 cells in hypoxia or normoxia were treated with control-NP or K5-NP. Levels of MCP-1 (A) and TNF- α (B) secreted into the culture media were analyzed by ELISA. Data are the mean $\pm$ SD; $n = 3$.

studies suggested that the antiangiogenic activity of K5 is through attenuation of ischemia-induced VEGF overexpression. In addition, it has been reported that K5 blocks the activation of HIF-1, which is proposed to be responsible for its inhibitory effect on VEGF expression.¹² Consistent with these studies, our results showed that K5-NP downregulated the VEGF expression induced by laser lesion. However, we demonstrated that K5-NP also downregulated the levels of β -catenin in the retina. Since the TCF/ β -catenin dimer is known to regulate VEGF transcriptionally, the decrease in the β -catenin level by K5-NP may represent another transcriptional mechanism responsible for its downregulation of VEGF expression.

Inflammation is a common complication in multiple diseases such as AMD and DR.²⁴ It is well established that oxidative stress and chronic inflammation in the RPE, Bruch's membrane, retina, and choriocapillaris are major pathogenic features of AMD.^{25,26} Multiple inflammatory factors are known to be overexpressed in the retinas with AMD and DR. Our results showed that K5-NP suppressed expression of TNF- α and ICAM-1, which are known to play important roles in leukocyte adhesion and infiltration. These findings indicate that K5 has an anti-inflammatory effect. Consistent with this, the result that K5-NP reduced retinal vascular leakage provided further support of its anti-inflammatory activity. In cultured retinal cells, K5-NP also downregulated the expression of MCP-1 and TNF- α , suggesting that K5's anti-inflammatory effect is exerted directly on retinal cells. Downregulation of NF- κ B by K5-NP may also contribute to its anti-inflammatory effect in the CNV model.

The receptor and signaling pathway mediating the anti-inflammatory activity of K5, however, remain to be defined.

Acknowledgments

The authors thank Anne Murray and Ying Chen for critical review of the manuscript.

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