Tissue Kallikrein Attenuates Choroidal Neovascularization via Cleavage of Vascular Endothelial Growth Factor

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Purpose. To investigate the antiangiogenic properties of tissue kallikrein in a murine model of laser-induced choroidal neovascularization (CNV).

Methods. CNV was induced in male C57BL/6J mice by laser photocoagulation. The animals received daily subcutaneous injections of tissue kallikrein (50 μg/kg) or vehicle control for 2 days before the laser photocoagulation, and this treatment continued until sample collection. Seven days after laser injury, the CNV size was quantified. The levels of monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1, and interleukin (IL)-6 were assessed by enzyme-linked immunosorbent assay 3 days after laser injury. Cleavage of mouse VEGF with tissue kallikrein was assessed in vivo and in vitro. The protein levels of bradykinin were assessed in the RPE-choroid complexes and hearts.

Results. A significant decrease in CNV size was observed in animals treated with tissue kallikrein (27,168.3 ± 2432.2 μm^2) compared with vehicle-treated controls (36,374.6 ± 3204.1 μm^2, $P < 0.05$). Tissue kallikrein treatment significantly reduced MCP-1, ICAM-1, and IL-6 levels in RPE-choroid complexes. Furthermore, immunoblotting showed the bands, presumably corresponding to the fragmented $VEGF_{164}$ protein, in the samples of both mouse VEGF preincubated with tissue kallikrein and RPE-choroid complexes obtained from animals treated with tissue kallikrein. In addition, bradykinin was unchanged in the RPE-choroid complexes of animals treated with tissue kallikrein, whereas the level of bradykinin was increased in the heart obtained from these experimental animals.

Conclusions. The current data indicate that kallikrein exhibits antiangiogenic properties by cleaving $VEGF_{164}$ in a laser-induced CNV model. (*Invest Ophthalmol Vis Sci.* 2013;54:274–279) DOI:10.1167/iovs.12-10512

Wet AMD is a leading cause of visual disturbance among people over the age of 50 years in the Western world[1] and is characterized by choroidal neovascularization (CNV), pathological angiogenesis originating from the choroid. Recent studies have identified VEGF as a key molecule in CNV formation; this has led to the development of therapeutic strategies for AMD using humanized anti-VEGF antibody fragments.[2] However, in recent years, there is widespread concern that long-term inhibition of all VEGF isoforms may cause ocular complications[3,4] and unexpected systemic consequences due to inhibition of the physiological roles of VEGF.[5,6]

Tissue kallikrein is a serine protease that contributes to bradykinin production and causes flow-dependent arterial dilation through activation of bradykinin B2 receptors coupled with endothelial nitric oxide release.[7] The kallikrein-kinin system (KKS) is one of the main mechanisms controlling systemic and local hemodynamics. In ocular tissues, it was previously demonstrated that administration of tissue kallikrein improved ophthalmic circulation by increasing chorioretinal blood flow.[8] In addition to the effect on ocular circulation, it has recently been reported that tissue kallikrein also possesses antiangiogenic effects through its enzymatic property of $VEGF_{164}$ cleavage, corresponding to the human $VEGF_{165}$ isoform, thereby reducing the pathological vascular changes in a murine oxygen-induced retinopathy model.[9] This led to the hypothesis that selective inhibition of $VEGF_{165}$ by tissue kallikrein is suitable for the maintenance phase of anti-VEGF therapy as opposed to continuous, repeated injections of anti-VEGF antibodies. However, no data are available thus far on the effects of tissue kallikrein on CNV formation.

In this study, we investigate the impact of tissue kallikrein in a laser-induced CNV model.

Materials and Methods

Experimental Animals and CNV Induction

Male C57BL/6J mice (8 weeks old; CLEA Japan, Tokyo, Japan) were used in this study. Animals were housed in plastic cages in a climate-controlled animal facility and were fed laboratory chow and water ad libitum. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

To generate CNV by laser injury, mice were anesthetized with 0.2 to 0.3 mL of 0.5% pentobarbital sodium, and pupils were dilated with 5.0% phenylephrine and 5.0% tropicamide. CNV was induced with a 532-nm laser (Novus Spectra; Lumenis, Tokyo, Japan). Laser spots (200 mW, 75 μm, and 100 ms) were created in each eye using a slit-lamp delivery system and a cover glass as a contact lens. The production of a bubble at the time of laser insult confirmed the rupture of Bruch's membrane.

Treatment

Animals were pretreated with a subcutaneous injection of rat urine tissue kallikrein (50 μg/kg), gifted by Sanwa Kagaku Kenkyusho Co., Ltd. (Mie, Japan), or PBS daily for 2 days before photocoagulation, and the treatments continued daily until the end of the study. To block

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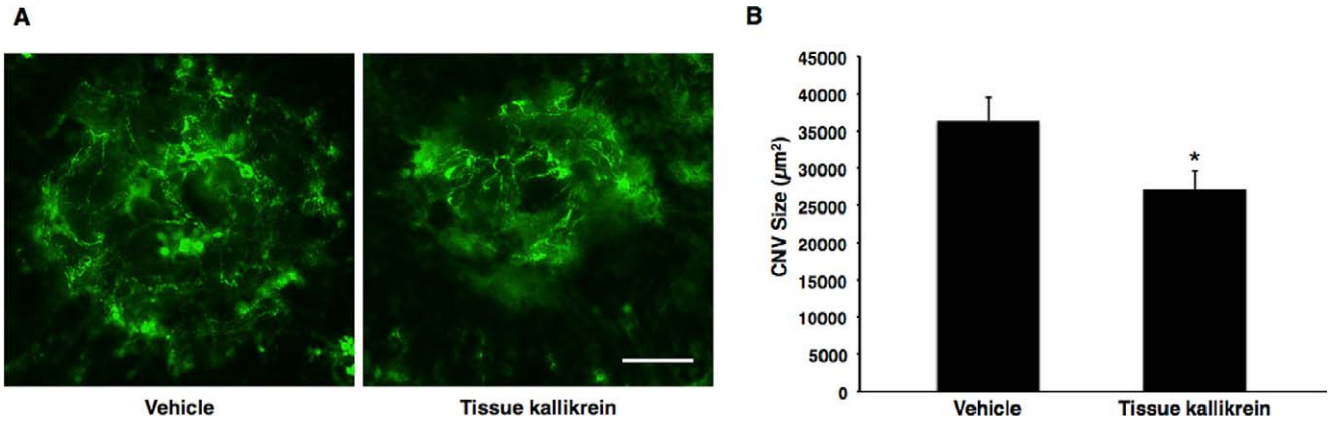

Figure 1. Impact of tissue kallikrein on CNV formation. (**A**) Representative micrographs of CNV lesions in the choroidal flatmounts from animals treated with either vehicle or tissue kallikrein. *Scale bar*: 50 μm. (**B**) Quantitative analysis of CNV size. *Bars* show the average CNV size in each group. Values are mean ± SEM ($n = 57$–66). $^*P < 0.05$.

VEGF, neutralization antibody for mouse VEGF (10 ng/eye; R&D Systems, Minneapolis, MN) was injected into the vitreous cavity using a 33-gauge needle (Exmire microsyringe; Ito Corporation, Tokyo, Japan) immediately after laser photocoagulation.

Choroidal Flatmount

Seven days after the laser injury, the size of the CNV lesions was quantified using a choroidal flatmount technique. Briefly, mice were anesthetized and perfused through the left ventricle with 5 mL PBS followed by 2 mL of 0.5% fluorescein-labeled dextran (Sigma-Aldrich, St. Louis, MO) in 1% gelatin. The eyes were enucleated and fixed in 2% paraformaldehyde for more than 30 minutes, and the anterior segment and retina were removed from the eyecup. Approximately 4 to 8 relaxing radial incisions were made, and the remaining RPE-choroidal-scleral complex was flatmounted with mounting medium (Vectashield Mounting Medium; Vector Laboratories, Burlingame, CA) and coverslipped. Flatmounts were examined with a fluorescence microscope (BIOREVO; Keyence, Osaka, Japan), and images of each CNV were digitally stored. The area of CNV-related fluorescence was measured by computerized image analysis with microscope software (BZ-II analyzer; Keyence). Eyes with hemorrhagic complications such as vitreous hemorrhage or subretinal hemorrhage caused by the laser irradiation were excluded from the evaluation.

ELISA

RPE-choroid complexes were carefully isolated from the animal eyes 3 days after photocoagulation, and solubilized in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors (Roche Applied Science, Indianapolis, IN). The protein levels of monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1, and interleukin (IL)-6 were determined with mouse MCP-1, ICAM-1, and IL-6 ELISA kits (R&D Systems), respectively, according to the manufacturer's protocols. Similarly, the protein levels of bradykinin in the RPE-choroid complexes and heart tissues were assessed using mouse bradykinin ELISA kits (Phoenix Pharmaceutical, Burlingame, CA). The tissue sample concentration was calculated from a standard curve and corrected for protein concentration.

Immunoprecipitation and Immunoblot Analysis for VEGF

Retinal tissue and RPE-choroid complexes were carefully isolated from the eyes 3 days after photocoagulation and homogenized in RIPA buffer supplemented with protease inhibitors. After preincubation of sonicated tissue extracts with protein-A beads (Roche Applied Science), rabbit anti-VEGF antibody (Thermo Fisher Scientific, Fremont, CA) was added and left overnight at 4°C with gentle mixing. The beads were washed with RIPA buffer, suspended in SDS sample buffer, and analyzed by 15% SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membrane by electroblotting, and immunoblot analyses were performed using the rabbit anti-VEGF antibody as previously described.[10]

In an in vitro setting, recombinant mouse $VEGF_{164}$ (100 ng/mL; R&D Systems) was preincubated with tissue kallikrein (50 μg/mL) at 37°C for 24 hours, and subsequently the samples were applied to immunoblot analysis for VEGF.

Real-Time PCR

The expression levels of *F4/80* in the RPE-choroid complexes during CNV formation were examined by real-time PCR. In brief, 3 days after laser treatment, the RPE-choroid tissues were obtained from eyes with or without tissue kallikrein treatment and homogenized in extraction reagent (TRIzol Reagent; Life Technologies, Carlsbad, CA). Reverse transcription was performed with RT-PCR (GoScrip Reverse Transcriptase; Promega, Madison, WI) and oligo dT(15) primer. Real-time quantitative PCR was performed using a master mix (GoTaq qPCR Master Mix; Promega); a real-time master mix (THUNDERBIRD Probe qPCR Mix; TOYOBO, Tokyo, Japan); and a real-time PCR system (StepOne plus System; Life Technologies). The probe (TaqMan) for *F4/80* was purchased from Life Technologies. The primers used for mouse hypoxanthine guanine phosphoribosyl transferase *(Hprt) 1* were: 5′-CAAACTTTGCTTTCCCTGGT –3′ and 5′- CAAGGGCATATCCAACAACA –3.′ *Hprt1* was used as endogenous control. Threshold cycle (C_T) was determined automatically and relative change in mRNA expression was calculated using the ΔΔCT values as previously reported.[11]

Statistical Analysis

All results are expressed as mean ± SEM with *n*-numbers as indicated. The Student's *t*-test was used for statistical comparison between groups. Differences between the means were considered statistically significant when the probability values were <0.05.

Results

Impact of Tissue Kallikrein on CNV Formation

To examine whether tissue kallikrein has an impact on CNV formation in mice, we quantified the CNV size in the flatmounts of RPE-choroid complexes from both control

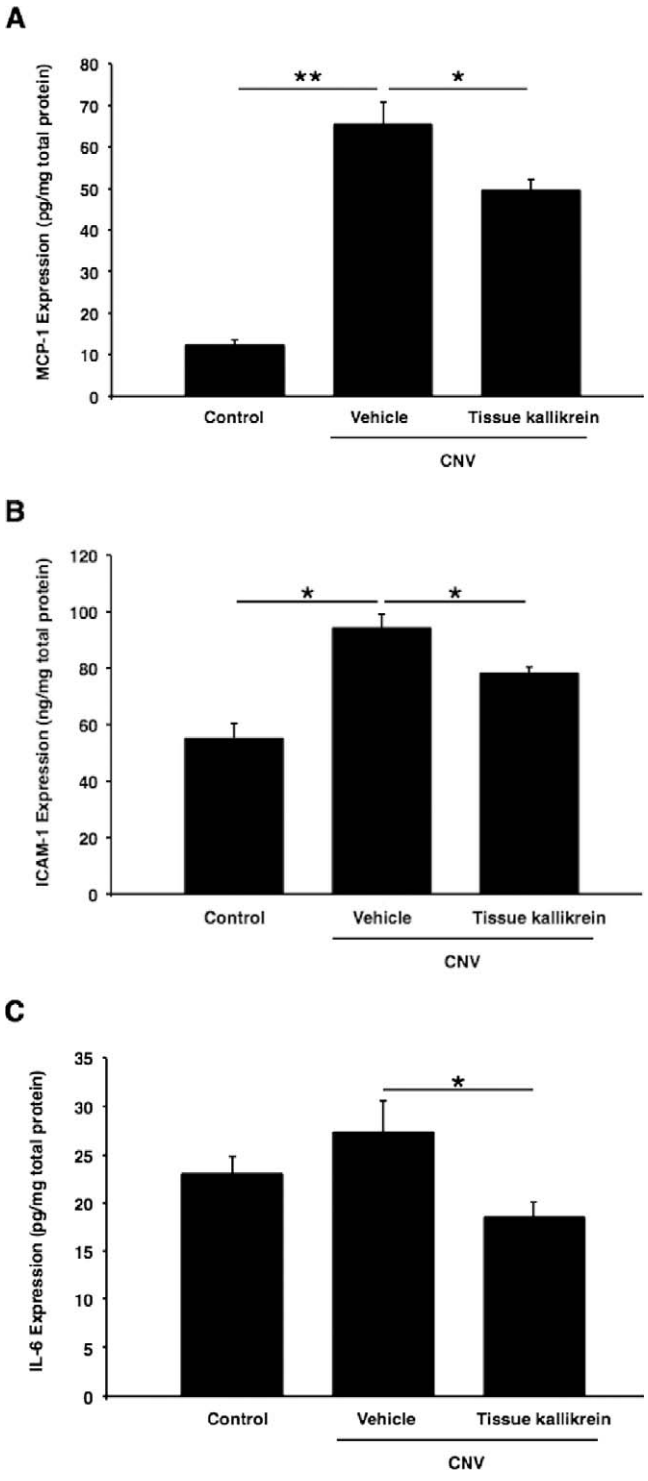

FIGURE 2. Impact of tissue kallikrein on the production of inflammation-associated molecules. *Bars* indicate the average protein levels of the elements under study in the RPE-choroidal complexes obtained from laser-induced CNV animals treated with vehicle or tissue kallikrein 3 days after laser photocoagulation, as measured by ELISA. (**A**) MCP-1. (**B**) ICAM-1. (**C**) IL-6. Values are mean ± SEM ($n = 6$–8 eyes). $^{*}P < 0.05$. $^{**}P < 0.01$.

animals and those that received tissue kallikrein treatment (Fig. 1A). Seven days after laser injury, a significant decrease in average CNV size was observed in animals treated with tissue kallikrein (27,168.3 ± 2432.2 μm², $n = 57$) compared with vehicle-treated animals (36,374.6 ± 3204.1 μm², $n = 66$, $P < 0.05$; Fig. 1B).

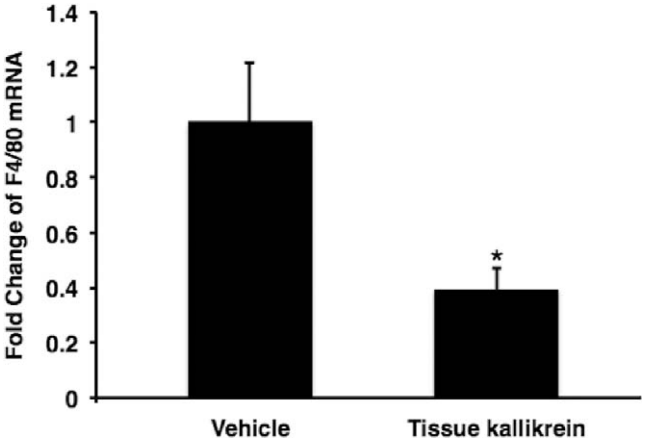

FIGURE 3. Impact of tissue kallikrein on macrophage infiltration during CNV formation. Relative *F4/80* mRNA expression normalized to the values of *Hprt* expression in the RPE-choroidal tissues obtained from animals with or without tissue kallikrein treatment. Values are mean ± SEM ($n = 4$–8 eyes). $^{*}P < 0.05$.

Reduction of Inflammatory Molecules and Macrophage Influx by Tissue Kallikrein

To investigate the mechanisms by which tissue kallikrein suppresses CNV formation, we measured the levels of inflammation-associated molecules, including MCP-1, ICAM-1, and IL-6, in the RPE-choroid complexes with or without CNV lesions 3 days after laser irradiation. Compared with protein levels of MCP-1 and ICAM-1 in the RPE-choroid complexes of normal animals (MCP-1, 12.3 ± 1.1 pg/mg, $n = 8$; ICAM-1, 55.5 ± 5.2 ng/mg, $n = 6$), these levels were significantly higher in animals with CNV (MCP-1, 65.5 ± 5.2 pg/mg, $n = 7$, $P < 0.001$; ICAM-1, 94.5 ± 4.6 ng/mg, $n = 6$, $P < 0.001$) at 3 days after laser injury (Figs. 2A, 2B). Protein levels of IL-6 were higher in the RPE-choroid complexes of animals with CNV (27.4 ± 3.1 pg/mg, $n = 7$) than in those of normal animals (23.0 ± 1.9 pg/mg, $n = 8$); however, the data did not reach statistical significance ($P = 0.25$, Fig. 2C). Nevertheless, protein levels of MCP-1, ICAM-1, and IL-6 were significantly reduced in the RPE-choroid complexes of the laser-treated animals that received tissue kallikrein compared with the vehicle controls (MCP-1, 49.6 ± 2.5 pg/mg, $n = 6$, $P < 0.05$; ICAM-1, 78.4 ± 2.2 ng/mg, $n = 6$, $P < 0.05$; IL-6, 18.6 ± 1.5 pg/mg, $n = 6$, $P < 0.05$, respectively; Fig. 2). In accordance with the reduction of inflammation-associated molecules, real-time PCR showed that *F4/80* mRNA expression was downregulated by 60.7% in the animals treated with tissue kallikrein ($n = 7$) compared with vehicle-treated animals ($n = 7$, Fig. 3), indicating a reduction in infiltrating macrophages in the CNV lesions.

Cleavage of $VEGF_{164}$ with Tissue Kallikrein

To explore the property of tissue kallikrein that attenuates CNV formation, we sought to determine whether tissue kallikrein interacts with $VEGF_{164}$, the isoform mainly relevant in pathological neovascularization. Firstly, mouse recombinant VEGF was preincubated with tissue kallikrein and applied to immunoblot for VEGF. Immunoblot analysis detected low molecular weight bands, which are not found in the samples without kallikrein treatment. In addition, a 16-kDa sample corresponding to fragmented $VEGF_{164}$ protein as described

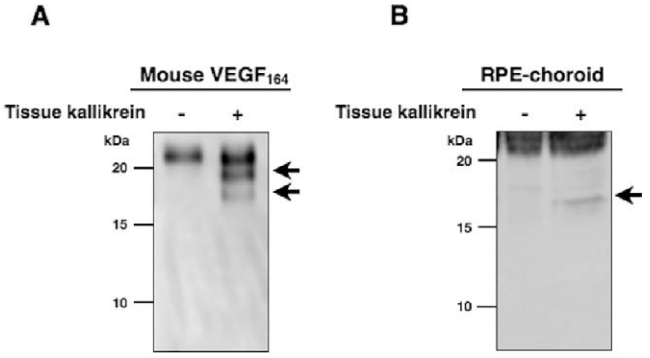

Figure 4. Cleavage of VEGF with tissue kallikrein in vivo and in vitro. (**A**) Immunoblot analysis for $VEGF_{164}$ preincubated with tissue kallikrein. (**B**) Immunoblot analysis for $VEGF_{164}$ in retinal and RPE-choroidal complex tissues. *Arrows* indicate fragment form of $VEGF_{164}$.

previously,[9] was detected in the samples of RPE-choroid complexes obtained from animals treated with tissue kallikrein, but not in the untreated control animals (Fig. 4). Both in vitro and in vivo data indicate that tissue kallikrein has properties to cleave and/or degrade the $VEGF_{164}$ isoform.

Bradykinin Production by Tissue Kallikrein in RPE-Choroid Complexes

To determine whether tissue kallikrein administration increases the production of bradykinin in RPE-choroid complexes, we measured bradykinin levels in RPE-choroid complexes of animals with or without tissue kallikrein administration. Bradykinin levels were higher in heart homogenates (1627 ± 171 pg/mg, $n = 8$) from animals treated with tissue kallikrein than in those from untreated animals (1063 ± 88 pg/mg, $n = 8$, $P = 0.037$) at 3 days after laser injury (Fig. 5A). In addition, bradykinin levels were higher in kidneys from animals treated with tissue kallikrein (546 ± 73 pg/mg, $n = 4$) than in those from the untreated controls (408 ± 21 pg/mg, $n = 4$); however, this difference did not reach statistical significance ($P = 0.157$, data not shown). Conversely, protein bradykinin levels in the RPE-choroid complexes of animals with CNV (100.5 ± 17.8 pg/mg, $n = 4$) were not significantly different from those in animals with CNV treated with systemic administration of tissue kallikrein (96.9 ± 13.4 pg/mg, $n = 4$, $P = 0.87$) at 3 days after laser injury, suggesting a privileged milieu for KKS in the eye (Fig. 5B).

Evaluation of Additive Effect of Tissue Kallikrein and VEGF Neutralizing Antibody on CNV Formation

To further determine whether systemic administration of tissue kallikrein exhibits an additive effect with anti-VEGF antibody on CNV formation in mice, we quantified the CNV size in the flatmounts of RPE-choroid complexes from both control animals and those that received tissue kallikrein treatment with or without VEGF neutralizing antibody. Seven days after laser injury and intravitreal injection of control IgG, animals treated with tissue kallikrein showed a significant decrease in average CNV size (24,493.4 ± 1442.8 μm^2, $n = 37$) compared with vehicle-treated animals (28,844.7 ± 1542.9 μm^2, $n = 40$, $P < 0.05$; Fig. 6), in concert with the data shown in Figure 1. However, in animals receiving intravitreal injection of anti-VEGF neutralizing antibody, there was no difference in CNV size between vehicle-treated (20,468.1 ± 826.4 μm^2, $n = 38$) and kallikrein-treated animals (19,956.0 ± 1325.6 μm^2, $n = 32$, $P = 0.744$; Fig. 6).

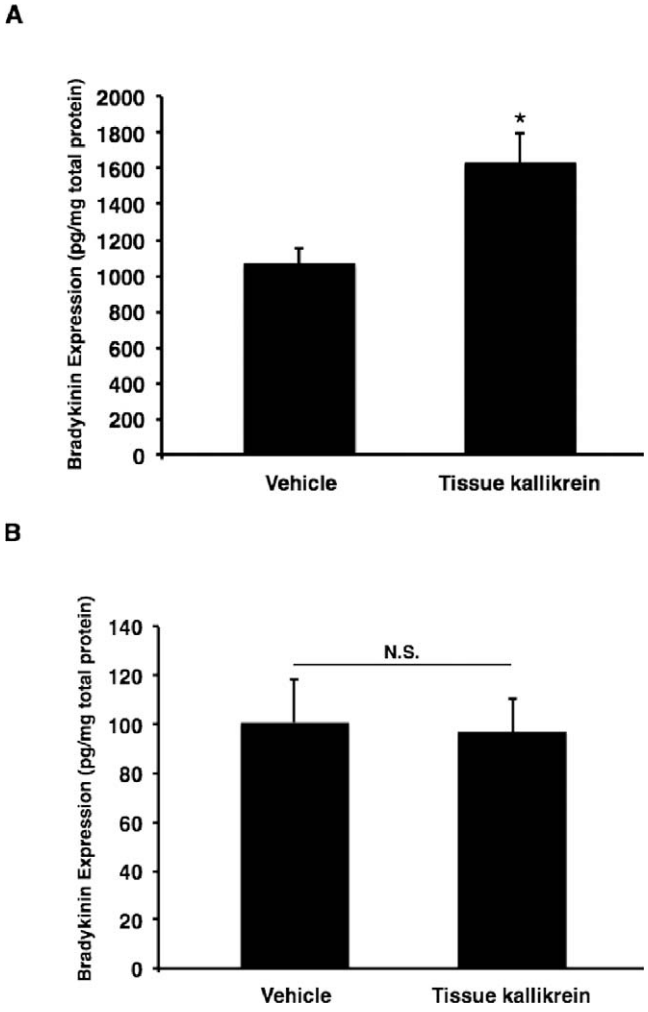

Figure 5. Production of bradykinin caused by tissue kallikrein administration. *Bars* indicate the average protein levels of bradykinin obtained from the animals treated with vehicle solution or tissue kallikrein. (**A**) Hearts. (**B**) RPE-choroid complexes. Values are mean ± SEM ($n = 4$–8 eyes). N.S., not significant. $^*P < 0.05$.

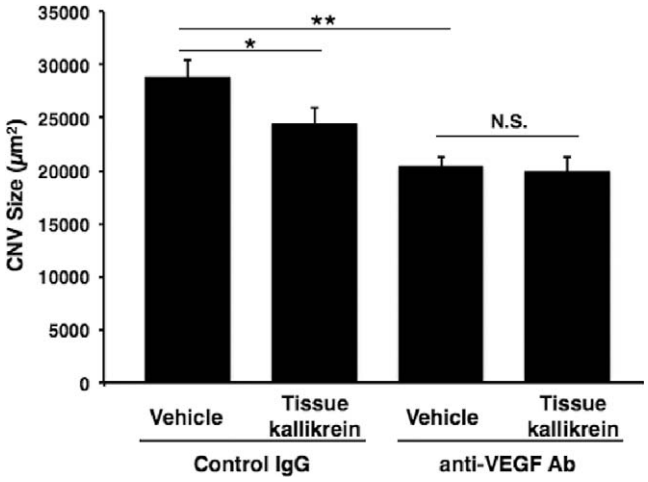

Figure 6. Evaluation of additive effect of tissue kallikrein and VEGF neutralizing antibody on CNV formation. Quantitative analysis of CNV size. *Bars* show the average CNV size in each group. Values are mean ± SEM ($n = 32$–40). $^*P < 0.05$. $^{**}P < 0.01$.

Discussion

In this study, we examined the ability of tissue kallikrein to suppress ocular angiogenesis in an animal model of CNV. Systemic administration of tissue kallikrein decreased the accumulation of inflammation-associated molecules and macrophage infiltration in the choroid during CNV formation, thereby reducing the size of the laser-induced CNV. Furthermore, the current data demonstrate that tissue kallikrein cleaves the VEGF isoform $VEGF_{164}$ in the RPE-choroid complexes. Alternatively, the concentration of bradykinin, known as a proangiogenic agent formed through KKS, was not altered in the RPE and choroid after tissue kallikrein treatment. The current data suggest a specific milieu for KKS in the eye and demonstrate the novel property of tissue kallikrein as an antiangiogenic factor for CNV.

Previous studies revealed that a variety of cytokines, chemokines, and endothelial adhesion molecules play pivotal roles in CNV formation.[12–15] In the current study, tissue kallikrein significantly decreased the protein levels of MCP-1 and ICAM-1, which are a potent macrophage-recruiting chemokine and a key endothelial adhesion molecule for leukocyte recruitment, respectively.[16,17] In accordance with these data, *F4/80* expression was reduced in the RPE-choroid complexes of animals treated with tissue kallikrein, indicating suppression of macrophage infiltration into the tissues. Targeted disruption of either MCP-1 or ICAM-1 genes in mice reportedly causes significant suppression of macrophage infiltration and subsequent attenuation of CNV development in experimental animal models[18,19]; therefore, the reduction of CNV formation by tissue kallikrein in our study appears to be, at least in part, due to the reduction of MCP-1 and ICAM-1 levels.

Next, to elucidate the mechanism by which tissue kallikrein suppresses ocular angiogenesis, we sought to determine whether tissue kallikrein interacts with VEGF, a key molecule of angiogenesis. Previously, it was demonstrated that tissue kallikrein cleaved $VEGF_{164}$ at the C-terminal domain and inhibited retinal neovascularization in an oxygen-induced retinopathy model.[9] In accordance with the previous reports, the current data showed the bands, representing putative digested products of $VEGF_{164}$ after tissue kallikrein treatment, both in vivo and in vitro. Since the biological activity $VEGF_{165}$ in humans is mediated by the C-terminal domain,[20] cleavage or degradation of $VEGF_{164}$ at the C-terminal domain by tissue kallikrein is likely to abrogate $VEGF_{164}$ function. VEGF is known to upregulate MCP-1 and ICAM-1 expression in cultured microvascular endothelial cells through NF-κB activation.[21,22] Therefore, it is likely that the decrease in VEGF by tissue kallikrein treatment might contribute to a lower inflammatory response induced by MCP-1 and ICAM-1, and thus smaller CNV lesions.

In this study, tissue kallikrein treatment also decreased the level of the inflammatory cytokine IL-6 in RPE-choroid complexes. It has been shown that VEGF activates mononuclear cells to produce IL-6,[23] suggesting that the decrease in VEGF by tissue kallikrein treatment might also contribute to a lower inflammatory response induced by IL-6. IL-6 is associated with tissue inflammation, which is crucial in CNV pathogenesis, via signal transduction and activation of transcription (STAT) 3 activation.[24,25] In addition, blockade of the IL-6 receptor or IL-6 genetic ablation was indeed reported to reduce MCP-1 and ICAM-1 production and subsequently suppress CNV.[12] Conversely, it has also been shown that IL-6 stimulates VEGF secretion in tumor cells via STAT3 pathways.[25] Taken together, the previous and current data suggest that tissue kallikrein interrupts the perpetual cascade of inflammatory events that exacerbates CNV formation by blocking $VEGF_{164}$, which in turn suppresses the production of associated molecules.

Notably, the KKS plays a central role in physiological and pathological processes, such as inflammation, coagulation, and vascular function.[26–28] Since tissue kallikrein produces kallidin, which eventually converts to bradykinin from low molecular weight kininogen, it is generally regarded that KKS contributes to proangiogenic reactions in addition to controlling systemic and local hemodynamics in vivo.[26] However, in our study, the concentration of bradykinin was unchanged in the RPE-choroid tissue between control animals and those that received tissue kallikrein treatment, whereas in other tissues, such as the heart and kidney, tissue kallikrein caused increases in bradykinin production. This indicates a specific milieu in ocular tissues for KKS function. In accordance with our data, it was previously demonstrated that the contribution of KKS to the pathogenesis of CNV was limited.[13] The previous and current studies suggest that tissue kallikrein predominantly may act as an antiangiogenic agent to cleave $VEGF_{164}$ with a minimal upregulation of bradykinin in ocular tissues.

Nonetheless, the use of tissue kallikrein as a treatment for CNV has yet to be carefully weighed. The current data showed no additive effect of tissue kallikrein with VEGF neutralizing antibody to suppress CNV development. It is most likely due to the potent effect of VEGF neutralizing agent to suppress CNV growth, and therefore tissue kallikrein could not be an alternative to the existing anti-VEGF agents to treat active CNV. The moderate effect of tissue kallikrein on VEGF suppression might be rather useful to maintain the status of treated CNV after the initial intervention with anti-VEGF agents (i.e., during maintenance phase). In addition, the effect of tissue kallikrein to improve choroidal circulation[8] might be beneficial to prevent CNV development, since it has been reported that decreased choroidal perfusion is a risk factor to develop CNV.[29,30] Further studies are still required to investigate the efficacy and safety of tissue kallikrein for treatment of CNV.

In conclusion, our data demonstrate that tissue kallikrein has an antiangiogenic effect by cleaving $VEGF_{164}$ in mouse eye. Since our study revealed that tissue kallikrein ameliorated CNV formation via systemic administration, tissue kallikrein could be a new approach for treating AMD.

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References

1. Jager RD, Mieler WF, Miller JW. Age-related macular degeneration. *New Engl J Med*. 2008;358:2606–2617.
2. Ip MS, Scott IU, Brown GC, et al. Anti-vascular endothelial growth factor pharmacotherapy for age-related macular degeneration: a report by the American Academy of Ophthalmology. *Ophthalmology*. 2008;115:1837–1846.
3. Murakami Y, Ikeda Y, Yonemitsu Y, et al. Inhibition of choroidal neovascularization via brief subretinal exposure to a newly developed lentiviral vector pseudotyped with Sendai viral envelope proteins. *Hum Gene Ther*. 2010;21:199–209.
4. Saint-Geniez M, Kurihara T, Sekiyama E, Maldonado AE, D'Amore PA. An essential role for RPE-derived soluble VEGF in the maintenance of the choriocapillaris. *Proc Natl Acad Sci U S A*. 2009;106:18751–18756.
5. Moorthy S, Cheung N. Cerebrovascular accidents and ranibizumab. *Ophthalmology*. 2009;116:1834–1835; author reply 1835.

6. Ueta T, Yanagi Y, Tamaki Y, Yamaguchi T. Cerebrovascular accidents in ranibizumab. *Ophthalmology*. 2009;116:362.
7. Madeddu P, Emanueli C, El-Dahr S. Mechanisms of disease: the tissue kallikrein-kinin system in hypertension and vascular remodeling. *Nat Clin Pract Nephrol*. 2007;3:208–221.
8. Yamaguchi T, Nagano H, Yamaguchi M, Suzuki T, Saito Y, Tano Y. The effects of kallidinogenase on choroidal blood flow in a hypertensive rabbit model. *Curr Eye Res*. 1999;18:417–422.
9. Nakamura S, Morimoto N, Tsuruma K, et al. Tissue kallikrein inhibits retinal neovascularization via the cleavage of vascular endothelial growth factor-165. *Arterioscler Thromb Vasc Biol*. 2011;31:1041–1048.
10. Murata M, Noda K, Fukuhara J, et al. Soluble vascular adhesion protein-1 accumulates in proliferative diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2012;53:4055–4062.
11. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402–408.
12. Izumi-Nagai K, Nagai N, Ozawa Y, et al. Interleukin-6 receptor-mediated activation of signal transducer and activator of transcription-3 (STAT3) promotes choroidal neovascularization. *Am J Pathol*. 2007;170:2149–2158.
13. Nagai N, Oike Y, Izumi-Nagai K, et al. Suppression of choroidal neovascularization by inhibiting angiotensin-converting enzyme: minimal role of bradykinin. *Invest Ophthalmol Vis Sci*. 2007;48:2321–2326.
14. Nagai N, Oike Y, Izumi-Nagai K, et al. Angiotensin II type 1 receptor-mediated inflammation is required for choroidal neovascularization. *Arterioscler Thromb Vasc Biol*. 2006;26:2252–2259.
15. Noda K, She H, Nakazawa T, et al. Vascular adhesion protein-1 blockade suppresses choroidal neovascularization. *FASEB J*. 2008;22:2928–2935.
16. Raoul W, Auvynet C, Camelo S, et al. CCL2/CCR2 and CX3CL1/CX3CR1 chemokine axes and their possible involvement in age-related macular degeneration. *J Neuroinflammation*. 2010;7:87.
17. Zen K, Parkos CA. Leukocyte-epithelial interactions. *Curr Opin Cell Biol*. 2003;15:557–564.
18. Sakurai E, Taguchi H, Anand A, et al. Targeted disruption of the CD18 or ICAM-1 gene inhibits choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2003;44:2743–2749.
19. Schmack I, Berglin L, Nie X, et al. Modulation of choroidal neovascularization by subretinal injection of retinal pigment epithelium and polystyrene microbeads. *Mol Vis*. 2009;15:146–161.
20. Keyt BA, Berleau LT, Nguyen HV, et al. The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. *J Biol Chem*. 1996;271:7788–7795.
21. Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem*. 2001;276:7614–7620.
22. Marumo T, Schini-Kerth VB, Busse R. Vascular endothelial growth factor activates nuclear factor-kappaB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. *Diabetes*. 1999;48:1131–1137.
23. Yoo SA, Bae DG, Ryoo JW, et al. Arginine-rich anti-vascular endothelial growth factor (anti-VEGF) hexapeptide inhibits collagen-induced arthritis and VEGF-stimulated productions of TNF-alpha and IL-6 by human monocytes. *J Immunol*. 2005;174:5846–5855.
24. Roh MI, Kim HS, Song JH, Lim JB, Koh HJ, Kwon OW. Concentration of cytokines in the aqueous humor of patients with naive, recurrent and regressed CNV associated with AMD after bevacizumab treatment. *Retina*. 2009;29:523–529.
25. Wei LH, Kuo ML, Chen CA, et al. Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. *Oncogene*. 2003;22:1517–1527.
26. Campbell DJ. The kallikrein-kinin system in humans. *Clin Exp Pharmacol Physiology*. 2001;28:1060–1065.
27. Moreau ME, Garbacki N, Molinaro G, Brown NJ, Marceau F, Adam A. The kallikrein-kinin system: current and future pharmacological targets. *J Pharmacol Sci*. 2005;99:6–38.
28. Phipps JA, Feener EP. The kallikrein-kinin system in diabetic retinopathy: lessons for the kidney. *Kidney Int*. 2008;73:1114–1119.
29. Boltz A, Luksch A, Wimpissinger B, et al. Choroidal blood flow and progression of age-related macular degeneration in the fellow eye in patients with unilateral choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2010;51:4220–4225.
30. Metelitsina TI, Grunwald JE, DuPont JC, Ying GS, Brucker AJ, Dunaief JL. Foveolar choroidal circulation and choroidal neovascularization in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2008;49:358–363.