

Observation of total VEGF level in hyperglycemic mouse eyes after intravitreal injection of the novel anti-VEGF drug conbercept

Liping Du,¹ Hui Peng,¹ Quan Wu,² Meidong Zhu,³ Delun Luo,² Xiao Ke,² Peizeng Yang,¹ Bo Lei¹

¹Ophthalmology, the First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology, Chongqing Eye Institute, Chongqing, China; ²Kanghong Biotech, Inc., Chengdu, China; ³Clinical Ophthalmology and Eye Health, Sydney Eye Hospital, University of Sydney, Save Sight Institute, Sydney, NSW, Australia

Purpose: Conbercept (KH902), a novel recombinant, soluble vascular endothelial growth factor (VEGF) receptor–IgG fusion protein, has been developed as a new drug for ocular neovascularization and macular edema. The present study aims to clarify the changes in conbercept levels, VEGF, and intraocular pressure (IOP) after the intravitreal injection of conbercept into diabetic mouse eyes.

Methods: Five-week-old *C57BL/6* mice were injected with streptozotocin to induce diabetes. Total VEGF and conbercept levels in the eyes were detected using an ELISA kit at –2 h, 1 h, 1 d, 4 d, 8 d, 16 d, 28 d, and 34 d after intravitreal injection of conbercept into diabetic and control mice. IOP was measured with a noninvasive TonoLab tonometer 7 d after intravitreal conbercept injection.

Results: The concentration of conbercept in the treated eyes increased immediately after injection and remained at high levels for 4 d (29.77±27.19 ng/ml, 20.28±28.85 ng/ml, and 42.43±36.51 ng/ml for days 1, 2, and 4, respectively). The concentration of conbercept in the untreated fellow eyes increased from day 2 to day 4 after injection with a level of about 1% of that in the injected eyes. Conbercept concentrations in both the treated and fellow eyes decreased from day 7 after intravitreal injection. The concentration of VEGF in the treated eyes increased significantly 1 h after injection when compared with the baseline measured 2 h before injection in both the diabetic and control mice (645.91±86.47 pg/ml versus 296.10±76.11 pg/ml and 860.50±201.47 pg/ml versus 377.69±70.72 pg/ml, respectively). VEGF concentration reached its peak 24 h after injection and then decreased thereafter. At day 7 after intravitreal injection, the difference in IOP between mice that received conbercept and mice that received PBS injections was not significant ($p>0.05$).

Conclusions: Conbercept and total VEGF levels in the mouse eyes were elevated after intravitreal injection of conbercept. Increased VEGF levels likely reflect VEGF sequestered by conbercept. These data could be helpful in understanding the metabolism of anti-VEGF drugs in the eye and for determining the protocol of multiple intravitreal injections of conbercept in patients.

Diabetic retinopathy (DR), a leading cause of vision loss worldwide, is a common and specific microvascular complication of diabetes [1]. Though the precise pathogenesis of DR must still be elucidated, it has been widely accepted that DR or proliferative DR is a vascular complication occurring in individuals with certain genetic backgrounds [2,3] and certain epigenetic modifications [4]. Visual dysfunction in DR patients is a direct consequence of the characterized retinal vascular lesions seen in these patients [5]. The vascular endothelial growth factor (VEGF) family, including VEGF-A to VEGF-E and the placenta growth factor (PlGF), plays an important role in angiogenesis by increasing vascular permeability to water, proteins, and other molecules. Studies have shown a positive correlation between the elevated intraocular VEGF in the vitreous and aqueous fluids and the severity of

DR or diabetic macular edema (DME) [6,7]. In addition, an increased VEGF receptor expression has been reported in the retinas from diabetic rats [8,9]. VEGF can be expressed by retinal-pigmented epithelial cells, astrocytes, vascular pericytes, endothelial cells, Müller cells, or retinal neurons, and it has been considered a stimulus for DME and choroidal neovascularization. Biologic agents targeting the VEGF have been shown to be effective in controlling DME and neovascularization caused by DR, age-related macular degeneration (AMD), and central retinal vein obstruction (CRVO).

These biologics, exerting their therapeutic role by antagonizing members of VEGF, can be classified into two categories: anti-VEGF drugs (including pegaptanib, ranibizumab, and bevacizumab) and soluble VEGF receptor decoys (such as aflibercept [10] and conbercept). Conbercept (also named KH902; 143 kDa) is a recombinant soluble VEGF receptor fused with the second immunoglobulin (Ig) domain of VEGF receptor 1 (VEGFR1), the third and the fourth Ig

Correspondence to: Bo Lei, Department of Ophthalmology, 1 You Yi Road, Yu Zhong District, Chongqing 400016 China; Phone: 86 23 89011851; FAX: 86 23 89011851; email: boleiy99@126.com

domains of VEGFR2, and the Fc region of human IgG [11,12]. Conbercept has a higher affinity with VEGF-A and all its isoforms by an additional fourth Ig domain of VEGFR2, which has been shown to be critical for the receptor dimerization and the enhancement of the association rate of VEGF to the receptor [11,12]. In addition, conbercept can bind to PlGF, to which biologics of anti-VEGF has no binding activity.

Clinical trials have demonstrated improvements in visual acuity, reductions in central retinal thickness, and decreases in CNV area in patients with AMD following multiple intravitreal injections of conbercept [12]. More recently, Huang et al. [11] has shown that conbercept could improve retinal function and inhibit the breakdown of the inner blood retinal barrier in streptozotocin (STZ)-induced diabetic rats. Although conbercept is becoming a novel potent agent for treating DME, AMD, and other retinal neovascularization conditions, the concentration of conbercept and its influence on the concentration of VEGF in the eyes of normal and hyperglycemic statuses remain unclear. Recently, it was shown that a single intravitreal injection of conbercept in rabbits reduced the ocular-free VEGF concentration over 60 d [13]. However, little is known about the concentration of conbercept and its influence on the concentration of VEGF in the eyes under normal and hyperglycemic statuses in mice, which is the most widely accepted animal model for the evaluation of retinal complications from STZ-induced diabetes. The present study aims to demonstrate the levels of conbercept in the mouse eye after a single intravitreal injection of conbercept, as well as the concentration of VEGF. The data are critical for understanding the metabolism of the drug and for determining protocols of multiple intravitreal applications.

METHODS

Animals: Five-week-old male *C57BL/6* mice were obtained from and housed in specific pathogen-free conditions in the animal center at Chongqing Medical University (Chongqing, China). All experimental procedures performed in this study complied with the ARVO statement for the use of Animals in Ophthalmic and Vision Research and were approved by the Ethics Committee in Animal and Human Experimentation of the First Affiliated Hospital of Chongqing Medical University.

Induction of diabetes in mice: In total, 112 mice were randomly assigned to the control ($n = 56$) and early diabetic groups ($n = 56$). Early diabetic mice were induced with an intraperitoneal injection of 60 mg/kg STZ (Sigma-Aldrich, St. Louis, MO) dissolved in a sodium citrate buffer (0.01M, pH 4.5) over 3 d, successively [14]. Type 1 diabetes mellitus

was confirmed by the glucose level in the tail vein blood measured by a glucometer. Fasting blood glucose levels higher than 250 mg/dl were considered early diabetic.

Intravitreal Injection: One microliter of 20 μ g conbercept was intravitreally injected into the right eyes of mice 2 weeks after the induction of diabetes. All procedures of intravitreal injection were performed under sterile conditions. First, the mice were anaesthetized by an intraperitoneal injection of 1% mebumal sodium and pupils were dilated. Both eyes were covered by sodium hyaluronate. Second, an aperture 1 mm posterior to the superotemporal limbus was made with a 30-gauge needle. A blunt 33-gauge needle was inserted through the opening, and injections (1 μ l of 20 μ g conbercept, provided by Chengdu Kanghong Biotech, Inc., or 1 μ l PBS) were given slowly over 30 s to allow for diffusion of the liquid. Tobramycin eye drops were topically administered on the treated eyes 3X per day in the week following intravitreal injection.

Intraocular pressure measurement: IOP was measured using the TonoLab rebound tonometer for rodents (Colonial Medical Supply, Franconia, NH) according to the manufacturer's recommended procedures [15]. In brief, mice were anaesthetized with an intraperitoneal injection of 10 ml/kg chloral hydrate (1%). The distance from the tip of the probe to the cornea of the eye was 1–4 mm. Mice received an intravitreal injection of conbercept (1 μ l, $n = 10$) or PBS (1 μ l, $n = 10$) in the left eyes. At 7 d after injection, IOP was measured noninvasively for these mice at 0 m, 5 m, 10 m, and 15 m after anaesthetization.

Preparation of homogenized fluid from mouse eye: All animals were euthanized by an overdose of carbon dioxide asphyxiation followed by cervical dislocation. Each vitreous body was removed, weighed, and kept in a sterile Eppendorf tube. Then, the vitreous bodies were immersed in a cell lysis buffer containing a 2% protease inhibitor cocktail and sonicated at 40 kHz on ice for 2 s, with a span of 10 s using an ultrasonic homogenizer (SONOPNCS, America). The homogenized fluid of the vitreous bodies were centrifuged at $3,000 \times g$ for 5 min at 4 °C and the supernatants were stored at –70 °C until the analysis.

VEGF and conbercept assays: VEGF and conbercept were measured by technicians blinded to the treatment. Total VEGF levels in the vitreous bodies were detected using a commercially available ELISA kit (Mouse VEGF Quantikine ELISA Kit, catalog number MMV00, R&D Systems) per the manufacturer's instructions. Briefly, 50 μ l of each of the standards (VEGF positive control), controls, and samples (10 folds dilution) were loaded onto a 96-well plate containing 50 μ l assay diluent in duplicate. The plate was incubated for

2 h at room temperature and then washed 5X. Then, 100 μ l of mouse VEGF conjugate solution was added, followed by incubation for 2 h at room temperature. After being washed 5X, 100 μ l of a substrate solution was added. After a 30-min incubation, 100 μ l of the stop solution was added, and the optical density was read at 450 nm using a microplate reader (VERSAmax, Molecular Device) with a correction wavelength of 540 nm. The VEGF concentration of each sample was calculated from the standard curve.

The free KH902 levels were measured by sandwich ELISA using mouse VEGF as the capture and mouse anti-human IgG Fc specific region antibody as the report. ELISA plates were coated with 100 μ l of 0.5 μ g/ml mouse VEGF (R&D Systems) at 4 °C overnight. Samples were diluted from 1:1 to 1:2,000, and the KH902 standard was diluted to 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, and 0 ng/ml. Samples and standards at 100 μ l were added to the plate and incubated for 1 h at room temperature. After being washed 3X with PBS/Tween, antigen-specific Ig G1 mouse antibodies were detected with 1:20,000 diluted HRP-conjugated mouse monoclonal anti-human IgG Fc antibodies (BETHYL). The A_{450} values were measured 30 min after substrate addition using a VERSAmax spectrophotometer (Molecular Devices).

Statistical analysis: A statistical analysis was undertaken using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Results are presented as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) followed by Bonferroni's correction were used for blood glucose, bodyweight IOP, concentrations of conbercept, and VEGF tests. Differences were regarded as significant when $p < 0.05$.

RESULTS

Body weight and blood glucose levels of early diabetic and age-matched control mice: The blood glucose level and bodyweight of early-diabetic and age-matched control mice ($n=56$ for each group) were measured at 1 and 2 weeks after injection of STZ. The early diabetic mice showed a significant increase in blood glucose (17.68 \pm 2.57 mg/dl and 23.12 \pm 3.16 mg/dl at 1 and 2 weeks respectively) in contrast to the controls (8.31 \pm 1.06 mg/dl and 8.47 \pm 0.99 mg/dl, $p < 0.001$, $p < 0.001$). When compared with the controls (18.38 \pm 0.95g and 19.72 \pm 1.06 g at 1 and 2 weeks respectively), a significant decrease in bodyweight (17.11 \pm 0.98 g and 17.45 \pm 0.85 g, $p < 0.001$, $p < 0.001$) was also observed in the early-diabetic mice (Figure 1).

IOP in early diabetic and age-matched control mice: An intravitreal injection of drugs may lead to an increase in IOP. The present study compared the IOP levels of eyes

injected with conbercept with the levels of those injected with PBS at 7 d after intravitreal injection. The levels of IOP in the conbercept-injected versus the PBS-injected mice were 11.63 \pm 1.71 mmHg versus 10.97 \pm 1.83 mmHg, 10.32 \pm 1.37 mmHg versus 10.83 \pm 1.50 mmHg, 9.53 \pm 1.05 mmHg versus 10.08 \pm 2.20mmHg, and 10.98 \pm 2.41 mmHg versus 10.37 \pm 2.27 mmHg at 0 min, 5 min, 10 min, and 15 min after anaesthetization, respectively (Figure 2). There was no significant difference in the level of IOP at any of the four times between the conbercept-injected and the PBS-injected eyes ($p > 0.05$).

Level of conbercept in eyes of normal mice after intravitreal injection: To clarify the level of conbercept in the eyes after intravitreal injection, 24 normal mice received an intravitreal injection of 20 μ g conbercept (1 μ l) in the right eye. The left untreated eyes served as controls. An ELISA assay showed that the concentration of conbercept in the treated eyes increased immediately after injection and remained at high levels for 4 d. The concentrations of conbercept assayed on days 1, 2, and 4 after intravitreal injection were 29.77 \pm 27.19 ng/ml, 20.28 \pm 28.85 ng/ml, and 42.43 \pm 36.51 ng/ml, respectively. For the contralateral control eyes, the concentration of conbercept increased significantly from days 2 to 4 after injection. The conbercept concentration in the contralateral untreated eyes was about 1% of that in the treated eyes. The concentrations in both eyes decreased at 7 d after intravitreal injection (Figure 3).

Level of conbercept in eyes of early diabetic mice after intravitreal injection: To clarify the level of conbercept in the eyes of early diabetic mice, 112 mice (56 early diabetic mice and 56 controls) received intravitreal injections of 20 μ g of conbercept (1 μ l) in the right eyes. The untreated left eyes served as controls. The concentration of conbercept was assayed for the homogenized fluid of the vitreous body harvested at -2 h, 1 h, 4 h, 8 h, 12 h, 1 d, 2 d, 4 d, 8 d, 12 d, 16 d, 22 d, 28 d, and 34 d after intravitreal injection with four animals in each group (Figure 4). The ELISA results showed that the concentration of conbercept in the vitreous bodies of both the early diabetic and control mice increased immediately after intravitreal injection and decreased steadily starting at day 4 after injection. Conbercept could not be detected starting from day 7 after intravitreal injection.

Level of VEGF in eyes of early diabetic mice after intravitreal injection of conbercept: As no antibodies were available to distinguish between the free and combined forms of VEGF, the total concentration of VEGF in the vitreous bodies (free VEGF plus presumably a combined form of VEGF, i.e., conbercept-VEGF) was assayed for the early diabetic and control mice at -2 h, 1 h, 1 d, 4 d, 8 d, 16 d, 28 d, and 34 d

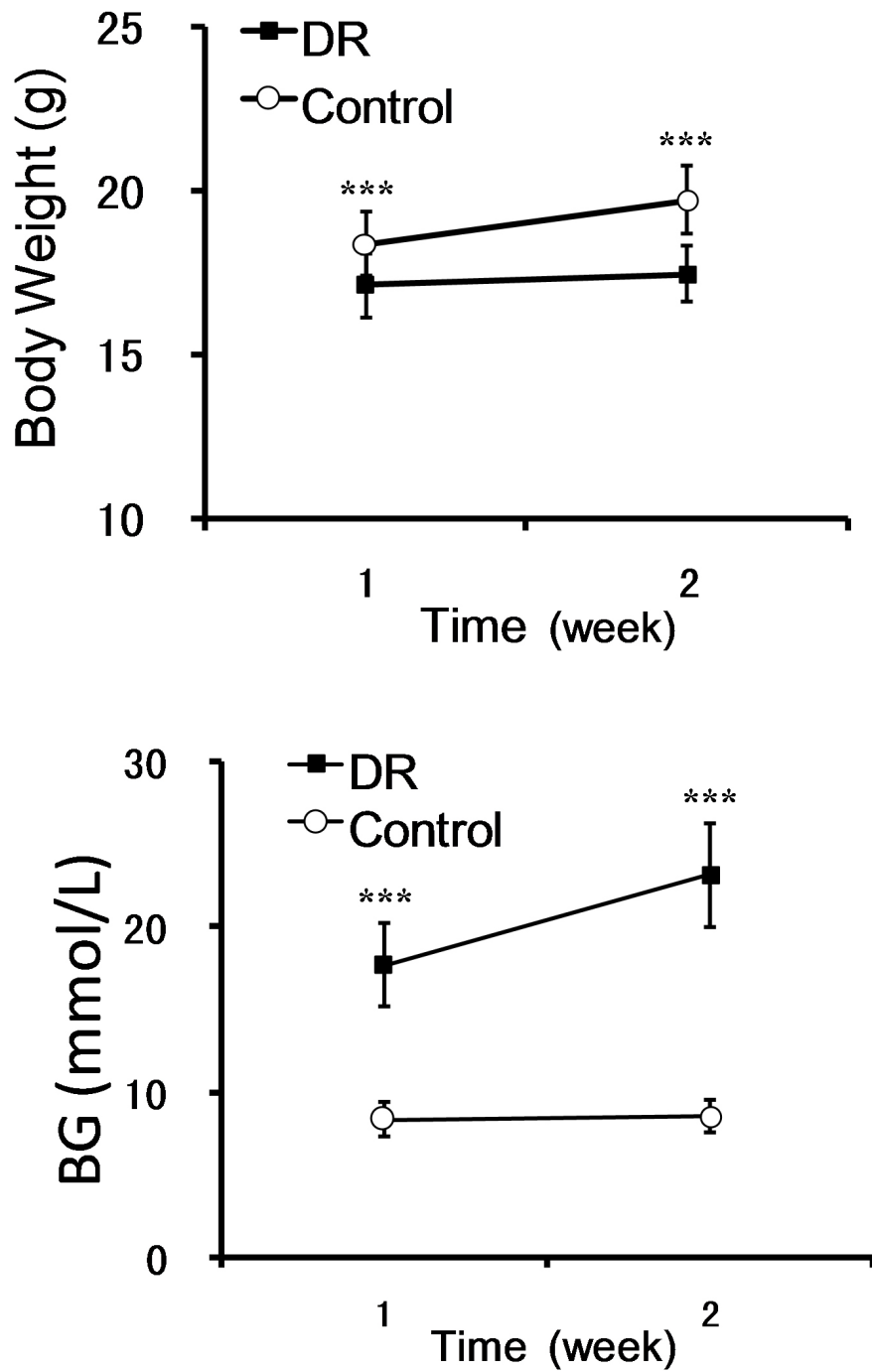


Figure 1. Body weight and blood glucose levels of diabetic and age-matched control mice. The left panel shows a decreased body weight of diabetic mice ($p < 0.001$, $p < 0.001$) and the right panel shows an increased level of blood glucose in diabetic mice ($p < 0.001$, $p < 0.001$) measured at 1 and 2 weeks after injection of STZ. One-way analysis of variance (ANOVA) followed by a Bonferroni correction was used for statistics. Values represents mean \pm SD ($n = 56$ for each group), *** $p < 0.001$.

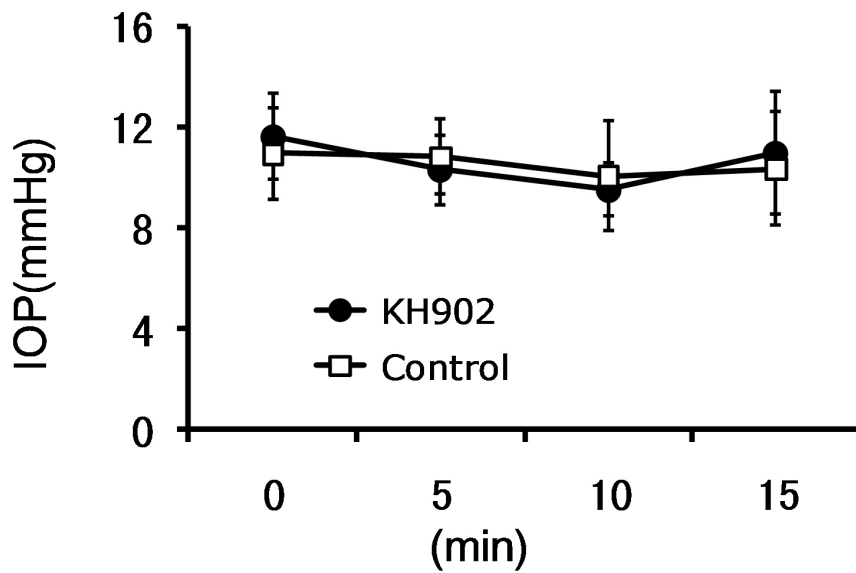


Figure 2. The level of IOP measured 7 d after intravitreal injection. There was no significant difference in the level of IOP between the conbercept (KH902)-injected and the PBS-injected eyes at 0 m, 5 m, 10 m, and 15 m after anaesthetization. One-way analysis of variance (ANOVA) followed by a Bonferroni correction was used for statistics. Values represents mean±SD. (n=10 for each group, p>0.05).

after intravitreal injection of conbercept with four animals in each group. The concentration of VEGF in the treated eyes was higher at 1 h after injection when compared with the pre-injection baseline in both the early diabetic and the control mice (645.91±86.47 pg/ml versus 296.10±76.11 pg/ml and 860.50±201.47 pg/ml versus 377.69±70.72 pg/ml, respectively). The VEGF concentration reached its peak 24 h after injection and then decreased steadily (Figure 5A).

To exclude the influence of the injection procedure on the total level of VEGF, the concentration of VEGF in eyes that received an intravitreal injection of PBS was also assayed. The concentration of VEGF was also higher 1 h after PBS injection in both the early diabetic and the control mice when compared with the pre-injection baseline (449.12±203.04 pg/ml versus 283.08±70.28 pg/ml and 539.59±181.64 pg/ml versus 381.76±108.90 pg/ml, respectively). As well, it

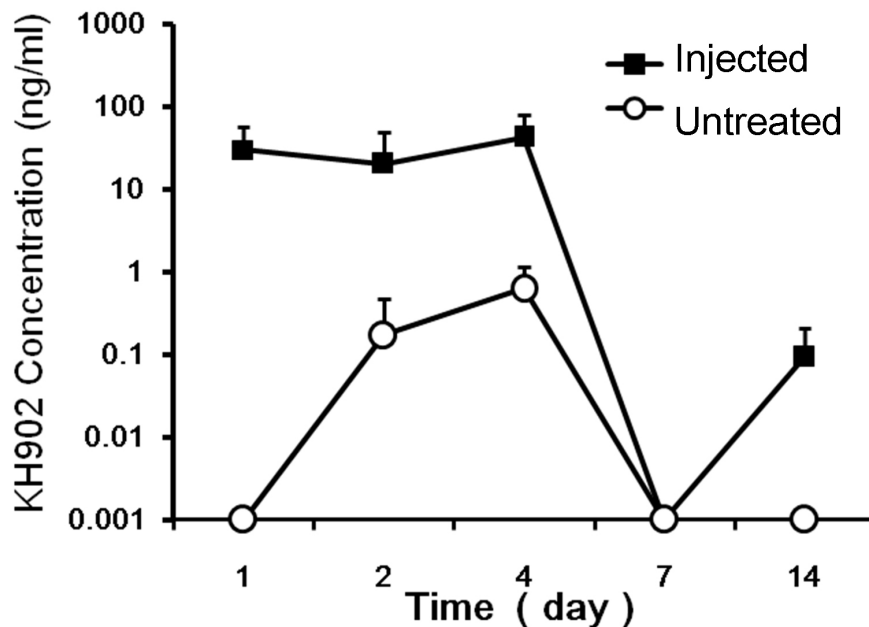


Figure 3. The concentration of conbercept in the treated and untreated contralateral eyes in normal mice. The level of conbercept increased immediately and remained at high levels during the first 4 d in the treated eyes and decreased to a normal level 7 d after intravitreal injection. The level of conbercept in the fellow eyes increased from 2 to 4 d after intravitreal injection. The conbercept concentration in the contralateral untreated eyes was about 1% of that in the treated eyes. Values represents mean±SD. (n=3 for each point).

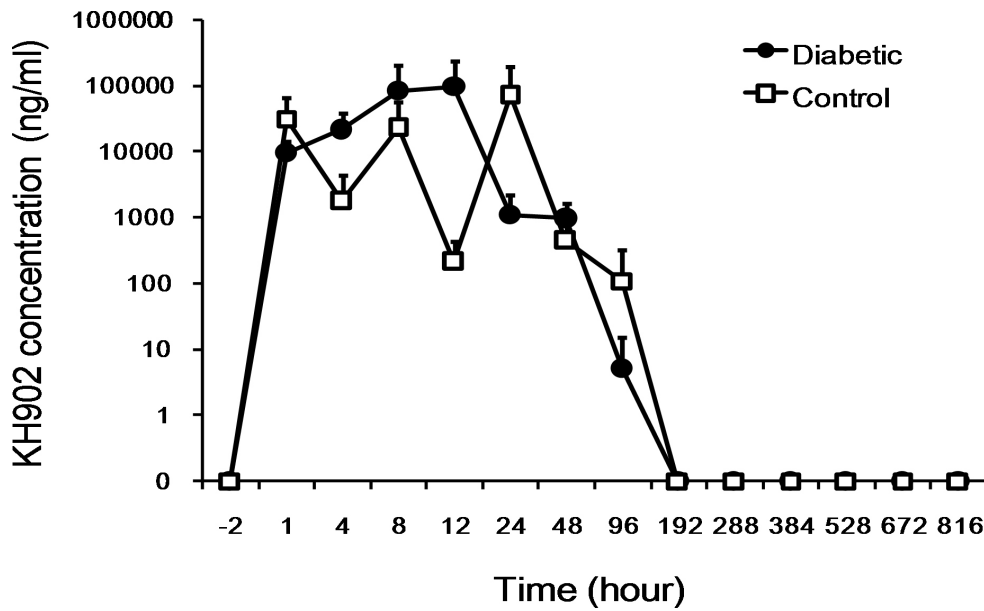


Figure 4. Level of conbercept (KH902) in eyes of diabetic mice after intravitreal injection. In both the diabetic and the control mice, the level of conbercept remained at high levels during the first 4 d after intravitreal injection. Conbercept could not be detected starting from day 7 after intravitreal injection. No significant was identified between the two groups at any time point. Values represents mean \pm SD. (n=4 for each point).

reached its peak 24 h after injection, though the differences were not statistically significant (Figure 5B). We normalized conbercept-mediated VEGF changes by subtracting the mean VEGF concentration in the PBS-injected eyes from the mean of the conbercept-injected eyes. After normalization, there was still an increase in VEGF concentration in the eyes of the early diabetic and control mice 1 h after conbercept injection (Figure 5C). The concentration of VEGF reached its peak 24 h after injection and then decreased steadily to the normal level 7 d after injection in both groups.

DISCUSSION

VEGF promotes ocular neovascularization as a potent endothelial cell mitogen [16], a chemotactic agent for bone marrow-derived endothelial cell precursors [17,18], a promoter of blood vessel extravasation [19], and a proinflammatory cytokine amplifying the secretion of VEGF [20,21]. It has been shown to play a major role in mediating active intraocular neovascularization in patients with ischemic retinal diseases, such as DR and retinal-vein occlusion [6]. Anti-VEGF therapy, introduced in the past two decades, offers a promising modality to patients with sight-threatening neovascularization and macular edema caused by AMD and DR [6]. However, for all the anti-VEGF biologics available, multiple injections are inevitable, and the protocol for intravitreal injections has been a focus of recent clinical trials. It is desirable to clarify the dynamic levels of these agents and VEGF in the vitreous body after intravitreal administration, because these data are crucial for the determination of the time intervals between multiple intravitreal injections.

We studied the concentrations of VEGF in vitreous bodies after intravitreal injections of conbercept, a novel soluble VEGF receptor decoy [22], in early diabetic mice. Our results showed that the concentrations of conbercept increased and maintained at high levels during the first 4 d after intravitreal injection. At the same time, conbercept was detected in the contralateral untreated eyes. More interestingly, in contrast with a previous study, which identified that a single intravitreal injection of conbercept in rabbits reduced ocular-free VEGF concentrations over 60 d [13], we found the total VEGF concentration increased following an intravitreal injection of conbercept. We showed that the concentration of conbercept remained at high levels during the first 4 d after intravitreal injection and was not detectable starting from day 7. Li and colleagues showed that conbercept lasted for over 81 d in rabbits [13]. The difference between the two studies could be explained by the fact that the mouse has much smaller eyes and the conbercept concentration in mouse eyes was below a detectable threshold. Similarly, due to the remarkable difference in size between a mouse eye and a human eye, the data obtained in this study could not be used directly to reflect what may happen in human subjects. Clearly, similar experiments are necessary in larger animals or in humans.

The therapeutic role of the VEGF receptor decoys on ocular neovascularization is due to the neutralization of VEGF and its isoforms. Surprisingly, we found the VEGF level increased in the eye after an intravitreal injection of conbercept. To clarify the source of the VEGF, we first assayed VEGF levels when PBS was injected into the eye.

We found a slight increase in VEGF in the eyes and proposed that the elevated VEGF could be induced partly by trauma caused by the injection procedure itself. The ELISA kit used in the present study detected the total VEGF concentration, including free VEGF and combined VEGF. Therefore, we presumed that the higher level of VEGF in the vitreous body after an injection of conbercept represented a composite of an increased level of combined forms of VEGF (VEGF plus conbercept) and free VEGF. Unfortunately, the total VEGF assayed in the present study could not distinguish the free VEGF from the combined VEGF. To understand whether such a combined form of VEGF exists and whether this form affects the distribution, the metabolism and excretion of VEGF are important in designing clinical protocols and interpreting the efficacy of the VEGF decoys. To our knowledge, there is still no report concerning the combined form of VEGF in the eye.

In addition to conbercept detected in the treated eyes for the first 4 d after intravitreal injection, we also detected conbercept in the fellow eyes in the first 2 d after injection, though the level of conbercept in the fellow eyes was only about 1% of that in the treated eyes. The route by which conbercept entered the fellow eyes is unknown. However, these results may partially explain improvements in the visual acuity of the untreated eyes in AMD patients who had received an intravitreal injection of anti-VEGF biologics in the contralateral eyes.

Intravitreal injections of drugs may lead to a transient increase in IOP [23], though the increased IOP does not usually require medical treatment. The present study showed that conbercept has no effect on IOP at day 7 after intravitreal injection.

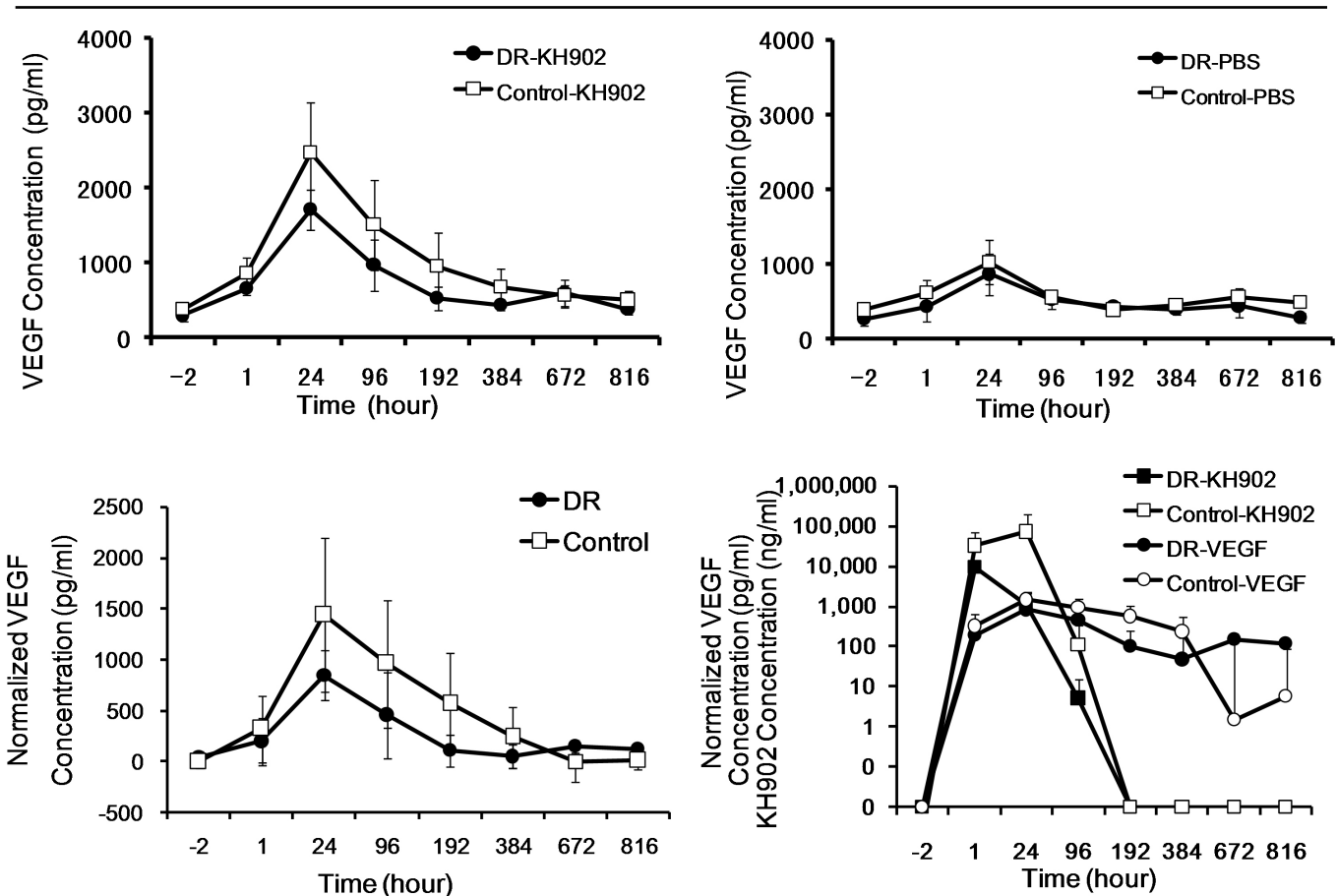


Figure 5. Level of the total VEGF (free and combined forms of VEGF) in eyes after intravitreal injection of conbercept (KH902). **A:** Level of VEGF in the eyes of both the diabetic and control mice after intravitreal injection of conbercept. **B:** Level of the total VEGF in the eyes of both diabetic and control mice after intravitreal injection of PBS. **C:** Normalized level of VEGF in eyes of both the diabetic and the control mice after intravitreal injection of conbercept. **D:** Conbercept and normalized VEGF concentrations in eyes of both the diabetic and the control mice after intravitreal injection of conbercept. One-way analysis of variance (ANOVA) followed by a Bonferroni correction was used for statistics. No significant was identified between the two groups at any time point. Values represents mean±SD. (n=4 for each point).

In conclusion, we showed increases in conbercept and total VEGF concentrations in the eyes after intravitreal injections of conbercept. The higher level of total VEGF could result from an increase in the combined form of VEGF, i.e., conbercept-VEGF. The data of the levels of conbercept and VEGF may be helpful in designing treatment protocol and in interpreting therapeutic effects.

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