The use of anti-vascular endothelial growth factor (anti-VEGF) agents has revolutionized the treatment of retinal vascular diseases associated with abnormal neovascularization or vascular permeability, including exudative age-related macular degeneration, diabetic macular edema, and macular edema secondary to retinal vein occlusion. Despite the success of potent anti-VEGF agents in treating diverse retinal disorders associated with the overproduction of VEGF problems such as short half-lives and a high injection frequency remain unresolved.

Three types of anti-VEGF antibodies are currently used for the treatment of age-related macular degeneration and retinal vascular disorders: the Food and Drug Administration-approved drugs ranibizumab and aflibercept, and the off-label drug bevacizumab. The molecular weights of bevacizumab (149 kDa) and ranibizumab (48.39 kDa) are considerably different because ranibizumab does not have a fragment crystallizable (Fc) region and bevacizumab is N-glycosylated in its Fc region. Aflibercept (VEGF Trap-Eye, Eylea) is not a monoclonal antibody, but a recombinant fusion protein consisting of portions of the human VEGF receptor (VEGFR) 1 and VEGFR2 extracellular domain fused to the Fc region of human IgG1. Generally, drugs with larger molecular weights are thought to...
have prolonged vitreous half-life.\textsuperscript{5} In fact, it was previously reported that the vitreous half-life of ranibizumab (2.75 days) was shorter than that of bevacizumab (7.06 days) and aflibercept (3.63 days) in rabbits.\textsuperscript{6–9}

Intravitreally administered anti-VEGF drugs reach first and in a larger proportion in the external layer (pericytes) and then reach the inner layer (endothelial cells) after crossing the blood–retina barrier.\textsuperscript{10} Recent studies reported that the elimination of intravitreally administered IgG across the blood–retina barrier into the systemic circulation is mediated by the neonatal Fc receptor (FcRn), which is expressed in the retinal pigment epithelium and endothelial cells of the retinal and choroidal vasculature.\textsuperscript{11–13} Ocular application of the Fc region and FcRn in intraocular pharmacokinetics (PK) is important in determining the duration of therapeutic efficacy of drugs as well as in anticipating the systemic exposure of intravitreally injected anti-VEGF agents, which can potentially cause systemic complications.

During the past few years, we have reported the intraocular PK of bevacizumab, ranibizumab, VEGF-Trap, and aflibercept in our previous study.\textsuperscript{8} Fc-VEGF-Trap was generated based on VEGF-Trap sequences. Instead of the Fc domain of VEGF-Trap, the amino acid sequence of the active region responsible for binding VEGF receptor–binding site.

Generation of Fc\textsubscript{VEGF-Trap}, a VEGF-Trap–Based Protein Obtained by Replacing the Fc Region of Human IgG1 With a Dimerized Coiled-Coil Domain

Previously, we generated VEGF-Trap, a fusion protein containing human VEGFR1-Ig2 and VEGFR2-Ig3 and the human Fc domain. (A) Fc\textsubscript{VEGF-Trap} contains a dimerized coiled-coil domain (AP-1, UniProtKB ID: P05412, 276R-314N) instead of the Fc domain in VEGF-Trap, which is a fusion protein containing human VEGFR1-Ig2 and VEGFR2-Ig3 and the human Fc domain. (B) Fc\textsubscript{VEGF-Trap} is composed of 309 amino acids, and the predicted molecular weight of Fc\textsubscript{VEGF-Trap} is two-thirds of that of VEGF-Trap. (C) The model structure of Fc\textsubscript{VEGF-Trap}/VEGF-A complex.

Materials and Methods

Animal Studies

This study was approved by the Seoul National University Bundang Hospital Institutional Animal Care and Use Committee, and all experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
A total of 42 eyes from 42 New Zealand White rabbits weighing approximately 1.5 to 2.0 kg were randomly assigned to VEGF-Trap (n = 21) or Fc/VEGF-Trap (n = 21) groups. The intraocular PK of VEGF-Trap and Fc/VEGF-Trap were evaluated using same experimental design as in our previous studies. 

Intramuscular injection of 15 mg/kg Zoletil (a mixture of tiletamine and zolazepam; Virbac Laboratories, Carros, France) and 5 mg/kg xylazine, and a topical ophthalmic anesthetic (1% proparacaine hydrochloride, Alcaine; Alcon Laboratories, Inc., Fort Worth, TX, USA) were used for anesthesia. Eyes under investigation were dilated with eye drops containing a mixture of phenylephrine and tropicamide. Povidone iodine solution (5%) was applied for periocular skin and conjunctival antisepsis. Next, all intravitreal injections of VEGF-Traps (0.3 mg/0.03 mL) or Fc/VEGF-Traps (0.2 mg/0.03 mL) were performed in the right eye. To administer the same molar dose of Fc/VEGF-Trap and VEGF-Trap, the dose of Fc/VEGF-Trap was matched to two-thirds of the dose of VEGF-Trap because the molecular weight of Fc/VEGF-Trap is two-thirds that of VEGF-Trap. A sterile 30-gauge needle was introduced 1 mm posterior to the surgical limbus in the superotemporal quadrant of the ocular globe. Three or four rabbits were killed at each of the following time points: 1, 24, 48, 120, 356, and 720 hours (1 hour and 1, 2, 5, 14, and 30 days) after injection. Enucleated eyes were immediately stored at −80°C until analysis. Before immunoassay, eyes were divided into vitreous, aqueous humor, and retina/choroid compartments. To solubilize the vitreous, samples were soaked in PBS with 1% BSA at 4°C overnight and centrifuged at 387g for 10 minutes. Tissue proteins were extracted from the lyastes of homogenized retina/choroids using lysis reagent (CellLytic MT, C3228; Sigma-Aldrich Corp., St. Louis, MO, USA) and a homogenizer.

**Drug Immunoassay**

We measured the concentration of Fc/VEGF-Trap in each compartment at serial time points by an indirect enzyme-linked immunosorbent assay (ELISA) in accordance with previous reports. 

The diluted 165 amino acid antigen solution of human recombinant VEGF (rVEGF) (10 μg/mL) in a 50 mM carbonate buffer (pH 9) was divided into aliquots in 96-well plates at 100 μL/well. The plates were incubated with rVEGF antigens at 4°C overnight, washed with PBS, blocked with PBS containing 1% BSA at 4°C for 4 hours, and dried at 4°C in an incubator. Vitreous, aqueous humor, and retina/choroid samples were diluted in PBS with 0.1% BSA, put into a 96-well plate, and incubated at 4°C overnight to form antigen and antibody (VEGF-Trap or Fc/VEGF-Trap) complexes. We incubated Strept-Tactin conjugated with horseradish peroxidase (HRP) (1:5000; Bio-Rad, Hercules, CA, USA, no. 1610381) to detect rVEGF/Fc/VEGF-Trap complex, and 1:20,000 goat anti-human IgG/Fc antibody labeled with HRP (Albcam, Cambridge, MA, USA) to detect rVEGF/VEGF-Trap, in 96-well plates for 2 hours at room temperature. 3.3, 3.5, 5.5'-Tetramethyl benzidine substrate was used to detect HRP activity, and the optical density of the color change, which reflects the VEGF-Trap or Fc/VEGF-Trap concentration, was determined using a standard ELISA plate reader (Bio-Rad) and embedded software, SoftMax Pro 5.4.1 (Molecular Devices, Sunnyvale, CA, USA). Using standard curves, the concentrations of VEGF-Trap and Fc/VEGF-Trap in our samples were calculated. The drug concentration in retina/choroid was defined as the weight of drugs (mg) to the weight of the retina/choroid tissue (g). 

**Pharmacokinetic Data Analysis**

Quantitative analysis of all samples was performed using a four-parameter logistic (4PL) curve, which is widely used in curve-fitting analysis for typical ELISAs, and is more reasonable for describing biological systems than linear curve or semilog plot. 

The changes in concentrations of VEGF-Trap and Fc/VEGF-Trap in the vitreous, aqueous humor, and retina/choroids were analyzed by one- and two-compartment models. The equations and detailed parameters used for pharmacokinetic modeling and analysis were described previously. 

The half-life of elimination (t½), mean residence time (MRT), observed maximum concentration (Cmax), area under the concentration–time curve (AUC), apparent volume of distribution (V/F), and apparent clearance (CL/F) were estimated. Using the AUC for each compartment, the total exposure of the aqueous humor and retina/choroid to VEGF-Trap and Fc/VEGF-Trap from the vitreous was calculated. Statistically, the mean values and standard deviation of drug concentration at each time point were calculated, and the estimated curves were plotted using these values. Estimated pharmacokinetic data are presented as parameter estimate (CV%) values. A low CV value indicates reliable parameter without large variability.

**RESULTS**

No adverse events or signs of ocular inflammation were observed after intravitreal injection of either VEGF-Trap or Fc/VEGF-Trap. The changes in estimates for Fc/VEGF-Trap in the vitreous, aqueous humor, and retina/choroids are shown in Table 2. The estimated concentration–time curves with observed concentrations at the six time points for VEGF-Trap and Fc/VEGF-Trap are shown in Figure 2. The concentrations of VEGF-Trap and Fc/VEGF-Trap in the aqueous humor and retina/choroids are shown in Table 3. The estimated half-lives of Fc/VEGF-Trap in the vitreous and retina/choroid were 145.02 and 102.12 hours, respectively. Similarly, the Cmax of both drugs in the aqueous humor and retina/choroid was reached at 1 hour (Table 2). The estimated half-lives of Fc/VEGF-Trap in the vitreous and retina/choroid were 1.59 and 2.30 times longer (145.02 and 102.12 hours, respectively) than those of VEGF-Trap (103.99 and 44.42 hours, respectively). The MRT of Fc/VEGF-Trap and VEGF-Trap was 209.22 and 150.02 hours, respectively. Likewise, the dose-normalized AUC of Fc/VEGF-Trap in the vitreous was 1.162 times higher than that of VEGF-Trap. In addition, the total exposure of the aqueous humor and retina/choroid to Fc/VEGF-Trap and VEGF-Trap was approximately 1.2% and 39% of the vitreous exposure, respectively, whereas VEGF-Trap concentrations in the aqueous humor and retina/choroid were approximately 25.2% and 26.2% of the vitreal exposure, respectively. These results indicate that the anterior excretion of Fc/VEGF-Trap is relatively low and the posterior excretion is relatively high, compared to that of VEGF-Trap, suggesting that Fc/VEGF-Trap shows a preference for posterior excretion. The V/F values of Fc/VEGF-Trap were higher than those of VEGF-Trap in the vitreous (5.34 vs. 4.45 mL) and the retina/choroid (22.84 vs. 15.25 mL), but not in the aqueous humor (22.26 vs. 15.62 mL). These results indicate that Fc/VEGF-Trap is mainly distributed in the posterior compartment.
TABLE 1. The Concentrations and Amounts of VEGF-Trap and Fc in the Vitreous, Aqueous Humor, and Retina/Choroid of Rabbit Eyes at 1 Hour and 1, 2, 5, 14, and 30 Days Post Injection

<table>
<thead>
<tr>
<th>Time</th>
<th>VEGF-Trap</th>
<th>FcVEGF-Trap</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitreous</td>
<td>Aqueous Humor</td>
</tr>
<tr>
<td>Conc, µg/mL</td>
<td>Amount, µg</td>
<td>Conc, µg/mL</td>
</tr>
<tr>
<td>1 h</td>
<td>3.44 ± 0.07</td>
<td>1.16 ± 0.04</td>
</tr>
<tr>
<td>1 d</td>
<td>3.74 ± 0.10</td>
<td>1.30 ± 0.07</td>
</tr>
<tr>
<td>2 d</td>
<td>3.53 ± 0.06</td>
<td>1.20 ± 0.05</td>
</tr>
<tr>
<td>5 d</td>
<td>4.83 ± 0.12</td>
<td>1.75 ± 0.09</td>
</tr>
<tr>
<td>14 d</td>
<td>5.93 ± 0.20</td>
<td>2.35 ± 0.15</td>
</tr>
<tr>
<td>30 d</td>
<td>6.83 ± 0.24</td>
<td>2.85 ± 0.18</td>
</tr>
</tbody>
</table>

Data are means ± SD. Conc, concentration; amount, total amount in the compartment.

DISCUSSION

In this study, we investigated and analyzed the ocular PK of an Fc region–free VEGF-Trap and compared it with that of VEGF-Trap. The lower molecular weight of the FcVEGF-Trap (compared to native VEGF-Trap) may promote initial elimination from the vitreous. However, the replacement of the Fc region with a dimerized coiled-coil domain may enhance the long-term intraocular retention of FcVEGF-Trap, which was found to be approximately 40% longer than that of the conventional VEGF-Trap in this study.

According to previous reports, molecular weight is one of the determinant factors for ocular PK. The rate of diffusion is approximately inversely proportional to the cube root of the molecular weight; therefore, high molecular weights are thought to prolong vitreous half-life. However, in our study, the low molecular weight FcVEGF-Trap showed a 1.39 times longer vitreous half-life than VEGF-Trap (145.02 vs. 103.99 hours) did. This suggests that the elimination of vitreous VEGF-Trap is not merely through a molecular weight–dependent mechanism and that Fc region is associated with the prolongation of intraocular half-life. Considering the smaller molecular weight of FcVEGF-Trap (100 kDa) compared to VEGF-Trap (145 kDa), the actual amount of FcRn-dependent elimination of VEGF-Trap is estimated to be larger than our result.

The elimination of an intravitreally injected drug is achieved through two main routes: anterior and posterior. Anterior elimination refers to the diffusion of vitreous drugs into the posterior chamber and turnover via aqueous and uveal flow. Posterior elimination is achieved through permeation across the posterior blood–eye barrier. The initial concentration (1 hour after intravitreal administration) of FcVEGF-Trap was relatively lower than that of VEGF-Trap in all three compartments, even allowing for the dosage difference between the two drugs to match the molar dose (Fig. 2). There was a sharp decrease in FcVEGF-Trap levels, which was more than that for VEGF-Trap, in the early time period. Although the exact mechanism for this sharp decrease could not be identified, it might be associated with the low molecular weight of FcVEGF-Trap. The low molecular weight FcVEGF-Trap could achieve early dynamic equilibrium, or a large amount of drug could be transported into the anterior chamber. After the rapid decline phase of drug concentration, a second phase was observed in which the relative concentration of FcVEGF-Trap was lower in the aqueous humor and higher in the retina/choroid than that of VEGF-Trap, suggesting that FcVEGF-Trap shows a preference for posterior excretion. Furthermore, the apparent Vd/F of FcVEGF-Trap, which may be correlated with the amount of drug distributed into the tissue, was significantly higher in the posterior compartment and lower in the anterior compartment, compared to that of VEGF-Trap. This indicates that FcVEGF-Trap is more readily distributed in the posterior segment of the eyeball than VEGF-Trap, whereas a larger portion of intravitreally injected VEGF-Trap is distributed in the anterior segment.

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tion possibly enhances the efficacy of Fc/VEGF-Trap by increasing the delivery of anti-VEGF molecules into the retina.

The common anti-VEGF agents, including bevacizumab and aflibercept, are Fc-containing proteins. The main receptors for these proteins are the Fcγ receptors (FcγR) on cell membrane and FcRn. The expression of FcγR has been previously demonstrated using an RPE model: the ARPE-19 cell line. FcγR is vital for increasing the serum circulating half-life of antibodies and for protecting them from lysosomal degradation. Hence, it is an important factor in systemic PK of antibodies. However, the function of FcRn in intraocular PK was not reported until recently, when studies showed the presence of FcRn on RPE cells; moreover, recent studies showed that FcRn, which is involved in intracellular uptake and transport of Fc-containing molecules in the retina, played an essential role in eliminating intravitreally administered IgGs across the blood–retina barrier into the systemic circulation. Despite recent studies regarding FcRn, the function of the Fc region in ocular PK remains controversial. Some reports showed that inhibition of FcRn increased apical to choroidal transport of bevacizumab, indicating a role of FcRn in the recycling of the molecule. Another study showed that protein molecular weight and Fc region did not play critical roles in ocular PK as they do systemically. According to our results, VEGF-Traps showed Fc region-dependent properties in ocular PK. Therefore, we suggest that the Fc region of VEGF-Trap may play a role in vitreoretinal-specific elimination of VEGF-Trap and that the Fc region is a potential factor that diminishes VEGF-Trap concentration in the posterior compartment of the eye. By considering these characteristics of VEGF-Trap associated with Fc region–dependent clearance, we can advance our understanding of ocular PK of anti-VEGF agents.

We generated a new Fc/VEGF-Trap by replacing for the Fc region of VEGF-Trap and that the Fc region is a potential factor that diminishes VEGF-Trap concentration in the posterior compartment of the eye. By considering these characteristics of VEGF-Trap associated with Fc region–dependent clearance, we can advance our understanding of ocular PK of anti-VEGF agents.

We generated a new Fc/VEGF-Trap by replacing for the Fc region of VEGF-Trap.

FIGURE 2. Concentration of Fc/VEGF-Trap and VEGF-Trap in the rabbit eyes. Points represent observed concentrations and lines represent estimated concentrations by models.

### Table 2. Estimated Pharmacokinetic Parameters of Fc/VEGF-Trap and VEGF-Trap After Intravitreal Injection Into Rabbit Eyes

<table>
<thead>
<tr>
<th>Drug</th>
<th>Compartment</th>
<th>$K_{oe}$ (Terminal) (h$^{-1}$)</th>
<th>$t_{1/2}$ (h)</th>
<th>MRT (h)</th>
<th>$C_{\text{max}}$ (µg/mL)</th>
<th>AUC (h·µg/mL)</th>
<th>$V_{d}/F$ (mL)</th>
<th>CL/F (mL/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fc/VEGF-Trap</td>
<td>Vitreous humor</td>
<td>0.0049 (26.11)</td>
<td>145.02 (26.09)</td>
<td>209.22 (26.09)</td>
<td>37.44 (8.05)</td>
<td>7252.22 (22.46)</td>
<td>10.03 (8.06)</td>
<td>0.262 (22.48)</td>
</tr>
<tr>
<td></td>
<td>Aqueous humor</td>
<td>0.0161 (76.24)</td>
<td>43.02 (76.17)</td>
<td>114.92 (328.86)</td>
<td>16.46 (8.38)</td>
<td>1032.68 (75.50)</td>
<td>22.26 (259.95)</td>
<td>0.194 (75.58)</td>
</tr>
<tr>
<td>200 µg</td>
<td>Retina/choroids</td>
<td>0.0068 (26.86)</td>
<td>102.12 (26.83)</td>
<td>234.40 (53.63)</td>
<td>13.83 (6.00)</td>
<td>2052.42 (25.97)</td>
<td>22.84 (32.02)</td>
<td>0.097 (25.99)</td>
</tr>
<tr>
<td>VEGF Trap</td>
<td>Vitreous humor</td>
<td>0.0067 (25.67)</td>
<td>103.99 (25.64)</td>
<td>150.02 (25.64)</td>
<td>67.37 (8.36)</td>
<td>10107.73 (21.69)</td>
<td>4.45 (8.36)</td>
<td>0.029 (21.71)</td>
</tr>
<tr>
<td>300 µg</td>
<td>Aqueous humor</td>
<td>0.0088 (57.55)</td>
<td>78.89 (57.50)</td>
<td>285.36 (183.96)</td>
<td>22.20 (5.79)</td>
<td>2546.34 (56.70)</td>
<td>33.62 (132.37)</td>
<td>0.118 (56.76)</td>
</tr>
<tr>
<td></td>
<td>Retina/choroids</td>
<td>0.0156 (58.11)</td>
<td>44.42 (58.06)</td>
<td>200.47 (161.33)</td>
<td>60.58 (5.47)</td>
<td>3944.30 (57.70)</td>
<td>15.25 (109.07)</td>
<td>0.076 (57.75)</td>
</tr>
</tbody>
</table>

Data are presented as parameter estimate (CV%). $K_{oe}$, elimination rate constant; $t_{1/2}$, half-life; MRT, mean residence time; $C_{\text{max}}$, observed maximum concentration; AUC, area under concentration-time curve; $V_{d}/F$, apparent volume of distribution; CL/F, apparent clearance.
Role of the FcRegion of Anti-VEGF Drugs

**Figure 3.** Vitreous concentrations of study drugs in the rabbit eye. This graph shows first-order eliminations of Fc/VEGF-Trap and VEGF-Trap, estimated from one-compartment models. Difference of slopes of the two plots implies that vitreous half-life of Fc/VEGF-Trap is longer than that of VEGF-Trap.

One of the limitations of our study is that the molecular weight of Fc/VEGF-Trap is lower than that of VEGF-Trap, and this may affect ocular PK, although these two drugs are predicted to have similar chemical properties. However, the longer half-life of Fc/VEGF-Trap despite its lower molecular weight emphasizes the role of Fc region in ocular PK. We replaced the Fc region with the coiled-coil domain, which was presumed not to affect the chemical properties of VEGF-Trap; but the coiled-coil domain itself might affect ocular PK by changing the chemical properties of the drug. Furthermore, we neither investigated the stability of Fc/VEGF-Trap nor performed a functional comparison of Fc/VEGF-Trap and VEGF-Trap. Despite these limitations, our study is the first to investigate the effect of Fc region on ocular PK by generating a novel molecule of Fc region–free VEGF-Trap.

In conclusion, Fc-region–deficient VEGF-Trap showed significantly longer vitreous and retina/choroid half-lives than conventional VEGF-Trap despite its lower molecular weight, indicating that Fc receptors in ocular tissues contribute to drug elimination. Our findings may be useful in future development of new anti-VEGF agents for intraocular administration. Truncation or mutation of the Fc region of protein drugs can prolong the intraocular residence time, reducing the number of injections and the systemic exposure to intraocular drugs.

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**References**


