Vascular Endothelial Growth Factor (VEGF) Concentration Is Underestimated by Enzyme-Linked Immunosorbent Assay in the Presence of Anti-VEGF Drugs

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Purpose. Commercially available enzyme-linked immunosorbent assay (ELISA) kits are often used to monitor vascular endothelial growth factor (VEGF) levels in exudative age-related macular degeneration. To test their accuracy, this study performed measurements using the ELISA kits in the presence of anti-VEGF drugs.

Methods. The concentrations of bevacizumab, pegaptanib, or ranibizumab at 28 days and aflibercept at 28 and 56 days after an injection were estimated based on previous pharmacokinetic studies. Vascular endothelial growth factor concentrations were measured with two widely used VEGF ELISA kits in the presence of anti-VEGF drugs or control mouse immunoglobulin G (IgG). The monocyte chemotactic protein-1 (MCP-1) ELISA kit was used as a non-VEGF ELISA control kit.

Results. The concentrations of aflibercept, bevacizumab, pegaptanib, and ranibizumab were estimated at 0.14 to 7.2, 4.9, 8.6, and 0.11 to 1.1 μg/mL, respectively. ELISA underestimated the VEGF concentration 2- to 100-fold lower in the presence of an anti-VEGF drug, except for pegaptanib, at all VEGF concentrations tested (7.8–1500 pg/mL). Vascular endothelial growth factor at 1000 pg/mL was measured as 92, 150, and 170 pg/mL in the presence of aflibercept (7.2 μg/mL), bevacizumab (4.9 μg/mL), and ranibizumab (1.1 μg/mL), respectively (all $P < 0.0001$), and the measured VEGF concentration decreased proportionately by 90% to 92% with aflibercept, 85% to 94% with bevacizumab, and 83% to 99% with ranibizumab. The control mouse IgG did not interfere with the measurement of VEGF. Ranibizumab did not affect the measurements with MCP-1 ELISA.

Conclusions. Investigators should exercise caution when interpreting measurements of VEGF ELISA in patients being treated with an anti-VEGF drug.

Keywords: measurement error, enzyme-linked immunosorbent assay, vascular endothelial growth factor, antivascular endothelial growth factor drug

Several studies have reported a dose-dependent reduction in the intraocular or serum concentration of vascular endothelial growth factor (VEGF) after intravitreal administration of anti-VEGF drugs.[1–3] All these studies, to the authors' knowledge, used commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits,[1–6] mostly Quantikine Human VEGF ELISA kits (R&D Systems, Minneapolis, MN, USA). Such sandwich ELISA kits use specific capture and detection antibodies. If anti-VEGF drugs bind to the same VEGF site that the ELISA kit antibodies do and are present in the analytic samples, competition between antibodies and drugs could introduce errors in VEGF concentration measurements (Figs. 1a–c). The Quantikine Human VEGF assay is designed to eliminate the interference; however, the interference cannot be completely avoided. In fact, interference from other VEGF-related factors, such as recombinant human VEGF receptors R1 and R2, is mentioned in the manufacturer's data sheet. We speculated that many anti-VEGF drugs could in fact interfere with VEGF measurements, and we tested this in vitro by performing a human ELISA assay with known concentrations of VEGF in the presence of an anti-VEGF drug (aflibercept, bevacizumab, pegaptanib, or ranibizumab) at therapeutic concentrations.

Methods

The Quantikine Human VEGF ELISA kit (R&D Systems) and the Invitrogen Human VEGF ELISA kit (Life Technologies, Camarillo, CA, USA) were evaluated for accuracy in the presence of anti-VEGF drugs. All assays were performed in triplicate according to each manufacturer's instructions. The VEGFs used were VEGF Standard (2000 pg/vial recombinant $VEGF_{165}$ in a buffered protein base with preservatives; lyophilized) for the Quantikine kit and Hu VEGF Standard (recombinant Hu VEGF-165 expressed in *Sf* 21 inset cells, containing 0.1% sodium azide) for the Invitrogen kit. Aflibercept, bevacizumab, pegaptanib, and

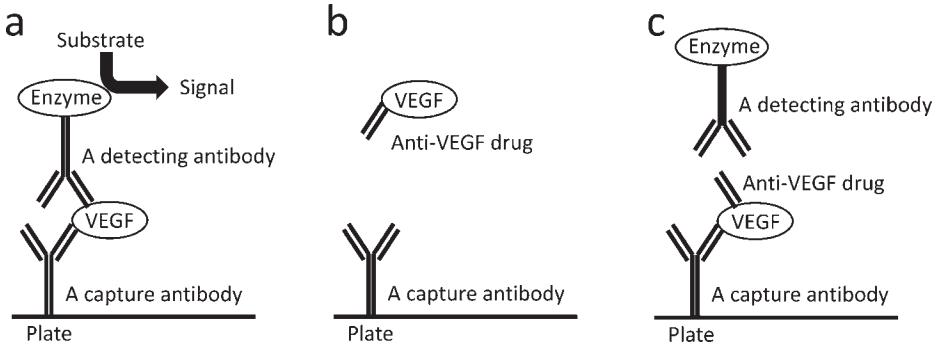

Figure 1. Possible mechanisms of interference by anti-VEGF drugs on VEGF ELISA measurements. (**a**) Schematic of the sandwich ELISA method. Capture antibodies and detection antibodies have different antigen-recognition sites. The capture antibody is bound to the plate. After adding sample containing VEGF and subsequent washing, the capture antibody and bound antigen remain on the plate. Then, an enzyme-linked detection antibody is added, followed by a wash, and finally by addition of an enzyme substrate that is converted to a detectable product in proportion to the antigen bound to the capture antibody. (**b**) If an anti-VEGF drug interferes with binding of the antigen to the capture antibody, some VEGF will be washed away, leading to underestimation of the actual concentration. (**c**) If an anti-VEGF drug instead interferes with antigen binding to detection antibodies, detection antibodies will be washed away, resulting in lower substrate conversion and underestimation of VEGF concentration.

ranibizumab were the anti-VEGF drugs tested, with mouse immunoglobulin G (IgG) used as a control. Vascular endothelial growth factor and anti-VEGF drugs were diluted with Calibrator Diluent RD5K (a buffered protein base with preservatives for cell culture supernatant samples) and standard diluent buffer (containing 0.1% sodium azide), respectively. Samples were incubated for 2 hours at room temperature for both kits. To exclude the possibility that these anti-VEGF drugs interfere with the ELISA assay nonspecifically, we assayed the effects of one of these agents, ranibizumab, in a non-VEGF ELISA assay that used monocyte chemotactic protein-1 (MCP-1), namely, the Quantikine Mouse MCP-1 ELISA kit (R&D Systems).

The capacity of the eye was defined as 4.5 mL; initial concentration of the anti-VEGF drug was calculated by dose/4.5 mL. Based on the previously investigated half-lives of these compounds,[7–11] the concentrations of aflibercept, bevacizumab, pegaptanib, and ranibizumab in the vitreous cavity were estimated following intraocular injection (28 and 56 days for aflibercept; 28 days for bevacizumab, pegaptanib, and ranibizumab).

Analytic samples were prepared by adding aflibercept, bevacizumab, pegaptanib, ranibizumab, or mouse control IgG to a solution containing a known concentration of VEGF: 7.8, 16, 31, 63, 125, 250, 500, or 1000 pg/mL for the Quantikine kit and 23, 47, 94, 188, 375, 750, or 1500 pg/mL for the Invitrogen kit. The detection limit of the human VEGF ELISA is 5.0 pg/mL according to the manufacturer's instructions. Ranibizumab (1.1 μg/mL) was also tested in an MCP-1 assay using the mouse MCP-1 ELISA kit. Free VEGF concentration was calculated using reported dissociation constants for each anti-VEGF drug.[8,12] Free VEGF concentrations were calculated using the definition of dissociation constant: $Kd = (A \times B)/AB$, where A, B, and AB are the concentrations of antigen, antibody, and antigen–antibody complex, respectively.

Concentrations were compared with the Student's t-test, and statistical significance was defined as 0.05.

Results

Determination of Concentrations of Anti-VEGF Drugs

Aflibercept. We estimated the concentration of aflibercept in the vitreous cavity 28 and 56 days after intraocular injection assuming a rabbit vitreal half-life of 4.8 days (unpublished data from Regeneron Pharmaceuticals, Tarrytown, NY, USA)[7] and used these concentrations ($2000/4.5 \times 0.5^{28/4.8} = 7.2$ and $2000/4.5 \times 0.5^{56/4.8} = 0.14$ μg/mL, respectively), as the aqueous concentration of aflibercept was not available in the literature at the time of this writing.

Pegaptanib. Rabbit aqueous concentrations of pegaptanib of 33.661, 11.086, 42.303, 23.889, and 0.338 μg eq./g were reported at 24, 96, 168, 312, and 1008 hours, respectively, after intravitreal pegaptanib injection (1.18 mg).[8] This yielded a concentration–time relation of $C(t) = e^{3.8414625 - 0.0046342t}$, which in humans equates to a concentration of $e^{3.8414625 - 0.0046342 \times 28} \times 1.625/1.18 = 8.6$ μg/mL at 28 days after injection.

Bevacizumab. After intravitreal injection of 1.25 mg bevacizumab in humans, peak aqueous concentration of bevacizumab was 33.3 μg/mL at day 1 and aqueous half-life was 9.82 days,[9] yielding a human concentration–time relation of $C(t) = 33.3 \times 0.5^{(t-1)/9.82}$ and therefore a concentration of $33.3 \times 0.5^{(28-1)/9.82} = 4.9$ μg/mL at 28 days after injection.

Ranibizumab. The concentration of ranibizumab was estimated based on two previous studies. One reported a mean concentration of 1.1 μg/mL in the aqueous humor of patients with retinal vein occlusion.[10] The other reported a half-life of 2.84 days in the anterior chamber of rabbit.[11] Based on the latter study, the concentration of ranibizumab would be only $500/4.5 \times 0.5^{28/2.84} = 0.11$ μg/mL at 28 days after injection. Given these disparate estimates, we investigated both concentrations in the current study.

Thus, we first tested the effects of the anti-VEGF agents at following concentrations: pegaptanib, 8.6 μg/mL; aflibercept, 7.2 and 0.14 μg/mL; bevacizumab, 4.9 μg/mL; and ranibizumab, 1.1 and 0.11 μg/mL. The control IgG was tested at 4.9 μg/mL, equivalent to that for bevacizumab.

Errors in ELISA-Measured VEGF Concentrations in the Presence of Anti-VEGF Drugs

The mean coefficient of variation (CV) of ELISA was 0.063, which was within the range of sample CVs listed in the kit's data sheet (0.035–0.065). The measured concentrations of VEGF were lower in the presence of anti-VEGF drugs ($P < 0.0001$) (Figs. 2b–d, 2f–h, 2k, 2l) than in the presence of mouse IgG ($P = 0.08$) (Fig. 2i) with the exception of pegaptanib ($P = 0.06$ and $P = 0.1$, respectively) (Figs. 2a, 2e), which did not interfere with VEGF measurements. When the known concentration of VEGF was 1000 pg/mL, the measured concentrations were 92 pg/mL with aflibercept, 150 pg/mL with bevacizumab, and 170 pg/mL

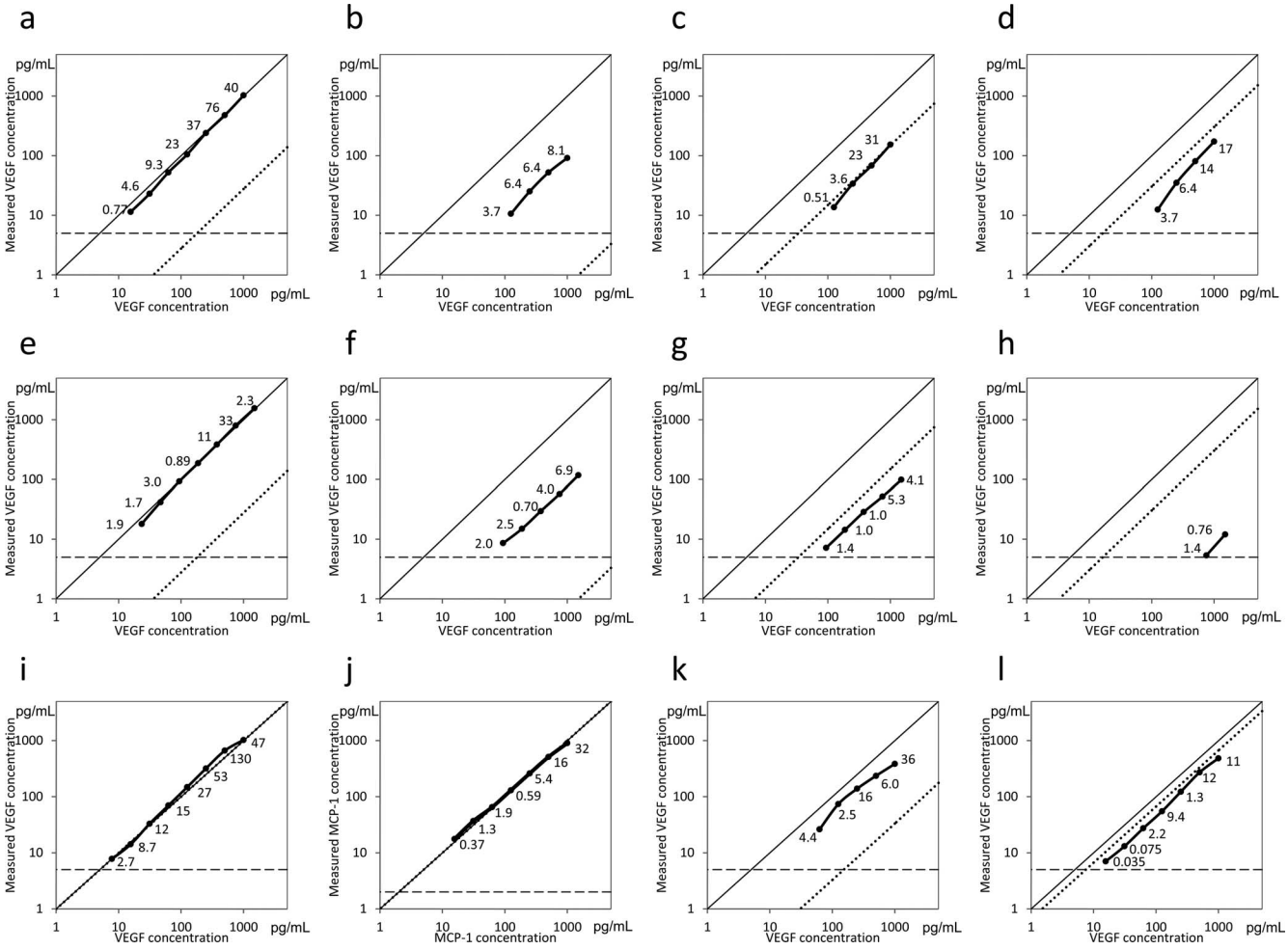

Figure 2. The concentrations of VEGF measured by the human VEGF ELISA kits were lower in the presence of anti-VEGF drugs than in the presence of a control mouse IgG. *Dotted lines* represent calculated free VEGF concentration, which did not match the measured VEGF concentrations. *Dashed lines* represent the detection limit. Assays were performed in the presence of the following: (**a**) pegaptanib (8.6 μg/mL), (**b**) aflibercept (7.2 μg/mL), (**c**) bevacizumab (4.9 μg/mL), or (**d**) ranibizumab (1.1 μg/mL) on a Quantikine Human VEGF ELISA kit (R&D Systems); (**e**) pegaptanib (8.6 μg/mL), (**f**) aflibercept (7.2 μg/mL), (**g**) bevacizumab (4.9 μg/mL), or (**h**) ranibizumab (1.1 μg/mL) on an Invitrogen Human VEGF ELISA kit (Life Technologies); (**i**) mouse IgG (4.9 μg/mL) on a Quantikine kit; (**j**) ranibizumab (1.1 μg/mL) on an MCP-1 measurement using an MCP-1 ELISA kit (Quantikine Mouse MCP-1 ELISA kit); or (**k**) aflibercept (0.14 μg/mL) or (**l**) ranibizumab (0.11 μg/mL) on a Quantikine kit. Data labels represent standard deviations (pg/mL).

with ranibizumab, which were all significantly lower than the known concentration of 1000 pg/mL (all $P < 0.0001$). The measured VEGF concentration decreased proportionately by 90% to 92% with aflibercept (7.2 μg/mL), 85% to 94% with bevacizumab (4.9 μg/mL), and 83% to 99% with ranibizumab (1.1 μg/mL). Measured VEGF concentrations were lower than calculated free VEGF concentrations in the presence of ranibizumab or bevacizumab (both $P < 0.0001$), except those measured by the Quantikine Human VEGF ELISA kit in the presence of bevacizumab ($P = 0.1$) (Fig. 2c). Conversely, measured VEGF concentrations were higher than calculated free VEGF concentrations in the presence of pegaptanib or aflibercept ($P < 0.0001$) (Figs. 2a, 2b, 2e, 2f, 2k). In contrast, ranibizumab did not significantly interfere with the measurement of MCP-1 using a mouse MCP-1 ELISA kit ($P = 0.2$) (Fig. 2j).

To further confirm the results and check the repeatability, another set of experiments was performed using different concentrations of anti-VEGF drugs (pegaptanib, 52 μg/mL; aflibercept, up to 440 μg/mL; bevacizumab, 4–14 μg/mL) and using the same concentrations of aflibercept (0.14 and 7.2 μg/mL) and ranibizumab (0.11–1.1 μg/mL) (Supplementary Fig. S1). The results were highly reproducible. Pegaptanib did not interfere with the measurements, whereas the measured VEGF concentration was much lower at higher concentrations of aflibercept, bevacizumab, and ranibizumab.

Discussion

The presence of the anti-VEGF drugs aflibercept, bevacizumab, and ranibizumab markedly reduced the VEGF concentration measured by ELISA compared with the known concentration. Ranibizumab did not inhibit a non-VEGF ELISA at a concentration that strongly interfered with VEGF ELISA analysis. Thus, the results confirmed our hypothesis that competition exists between anti-VEGF drugs (ranibizumab, bevacizumab, or aflibercept) and ELISA antibodies, except for pegaptanib.

Potential Competition Between Anti-VEGF Agents and ELISA Antibodies

Vascular endothelial growth factor$_{165}$ is composed of a receptor-binding region and a heparin-binding domain, among

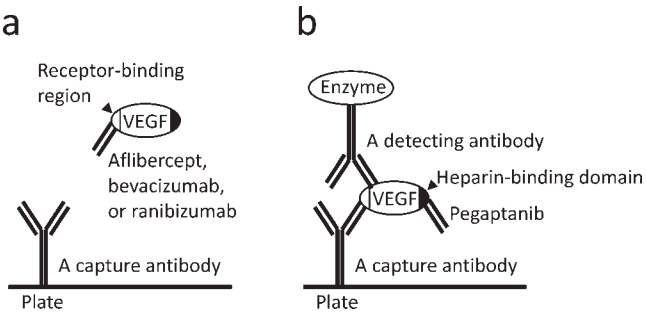

FIGURE 3. Possible mechanisms of interference by aflibercept, bevacizumab, ranibizumab, and pegaptanib. (**a**) Aflibercept, bevacizumab, and ranibizumab bind to a receptor-binding region of VEGF. If capture antibodies bind to the receptor-binding region, anti-VEGF antibody drugs interfere with the capture antibodies, leading to underestimation of the actual concentration. (**b**) Pegaptanib binds to a heparin-binding domain of VEGF. If capture antibodies bind to some regions other than the heparin-binding domain, pegaptanib does not interfere with the capture antibodies and the measured concentration will be accurate.

others. The binding sites of bevacizumab and ranibizumab are in the receptor-binding region,[12] while aflibercept binds to the receptor-binding region of VEGF[13] and pegaptanib binds to the heparin-binding domain.[14] The sandwich ELISA kits used in the current study use a monoclonal antibody as a capture antibody and a polyclonal antibody as a detection antibody. The binding sites of the antibodies used in these ELISA kits are not disclosed. However, given that the measured concentration was underestimated in the presence of ranibizumab, bevacizumab, or aflibercept, it is likely that the antibodies used in the ELISA kits bind to the receptor-binding region, although pegaptanib did not affect the measured concentration (Fig. 3).

Difference in the Extent of Errors Among Anti-VEGF Agents

Aflibercept has a dissociation constant of 0.49 pM, which is much lower than ranibizumab (46 pM), pegaptanib (49 pM), and bevacizumab (58 pM).[8,12] When considering binding constants only, the measured concentration of VEGF in the presence of aflibercept would be expected to be much lower than that in the presence of ranibizumab, but the measured VEGF concentrations were in fact similar in the presence of 0.14 μg/mL aflibercept and 0.11 μg/mL ranibizumab. This is presumably because anti-VEGF drugs have diverse molecular structures and may interfere with the ELISA analysis through different mechanisms (Figs. 1, 3). It is therefore conceivable that the extent of interference does not always reflect the anti-VEGF drug dissociation constant.

Clinical Significance of the Current Study

Based on the current results, investigators should be aware of the potential inaccuracy of VEGF ELISA kits when residual anti-VEGF drugs are present in the analytic sample. It is clear that certain anti-VEGF drugs reduce the estimated VEGF concentration. Measured VEGF concentrations in the presence of anti-VEGF drugs do not reflect the "biological activity" of VEGF,[15] either, for the clear reason that the capture antibody used in the kit does not mimic the VEGF receptor. Furthermore, the ELISA does not measure the free VEGF concentration, with the exception of the measurement using Quantikine Human VEGF ELISA kit in the presence of bevacizumab.

Limitations

There are several limitations to this study. The first is that the concentrations of anti-VEGF drugs used were based on half-lives or pharmacokinetic equations derived from different samples, such as from the anterior chamber or vitreous body of humans or rabbit. However, reported concentrations in actual patients have shown large variation, for example, from 0.0019 to 5.5 μg/mL.[10] Moreover, serum concentrations of anti-VEGF drugs after intraocular administration have not been measured. Second, we provide no alternative quantitative assays to yield more accurate VEGF concentration estimates. However, Western blot can be used only when the concentration of VEGF is high.[16]

CONCLUSIONS

The VEGF concentration measured by ELISA was lower than the actual VEGF concentration in the presence of the anti-VEGF drugs aflibercept, bevacizumab, and ranibizumab, particularly at high anti-VEGF drug concentrations. The measured VEGF concentration was not the same as the free VEGF concentration. Caution should be exercised when interpreting results of VEGF ELISA measurements in the presence of anti-VEGF drugs.

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