Bevacizumab and Aflibercept Activate Platelets via FcγRIIa

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Purpose. To confirm the formation of a drug–growth factor complex and investigate the effects of three VEGF inhibitors in the activation of platelets.

Methods. Growth factors and individual drugs were mixed and incubated. Scattered light intensity was measured by dynamic light scattering (DLS) to monitor the formation of drug–growth factor complex. Blood samples were obtained from 16 subjects (5 AMD patients and 11 healthy volunteers). Platelets obtained from the platelet-rich fraction by centrifugation were washed and resuspended in HEPES/Tyrode buffer. Platelet aggregability was assessed using a light transmission aggregometer in the presence of VEGF inhibitors and growth factors.

Results. In DLS study, a mixture of bevacizumab and VEGF-A showed one peak with a relatively gentle slope, indicating a heterogeneous mixture of multimeric bevacizumab–VEGF-A complexes. In aggregation study, no detectable aggregation was observed in the presence of ranibizumab, while significant aggregation was observed in the presence of VEGF-A and bevacizumab in five cases (one AMD patient and four healthy volunteers), VEGF-B and aflibercept in two cases (two volunteers), and placental growth factor (PlGF) and aflibercept in one case (one volunteer). No aggregation was observed when FcγRIIa antibody was added beforehand.

Conclusions. A complex composed of bevacizumab or aflibercept, but not ranibizumab, and growth factors activates platelets via FcγRIIa.

Keywords: bevacizumab, ranibizumab, aflibercept, FcγRIIa, platelet, thrombosis

Vascular endothelial growth factor (VEGF) inhibitor drugs bevacizumab, ranibizumab, and aflibercept are widely used in the treatment of neovascular AMD and other diseases, such as macular edema due to retinal vein occlusion, diabetic macular edema, and choroidal neovascularization due to pathologic myopia.[1–5] The use of these drugs has drastically increased in recent years.[6] Bevacizumab (Avastin; Genentech, Inc., South San Francisco, CA, USA) is a recombinant, humanized monoclonal antibody that binds all isoforms of VEGF-A.[7] It is used as an off-label treatment via intravitreal injection because the drug has target specificity similar to that of the on-label drug, ranibizumab (Lucentis; Genentech, Inc.), and is available at low cost.[2,8] Ranibizumab was specifically developed for intravitreal injection and has been approved for the treatment of neovascular AMD,[9,10] myopic choroidal neovascularization, diabetic macular edema, and macular edema secondary to branch- and central-retinal vein occlusion (BRVO, CRVO) in the United States and other countries including Japan. Aflibercept (Eylea, Regeneron Pharmaceuticals, Inc., NY, USA) is a soluble decoy receptor, comprising the second immunoglobulin (Ig) domain of human VEGFR-1 and the third Ig domain of human VEGFR-2 expressed as an inline fusion with the constant region (Fc) of human IgG1.[11] Aflibercept binds multiple isoforms of VEGF-A and related VEGFR-1 ligands, namely, VEGF-B and PlGF.[12]

Bevacizumab is known to increase the risk of arterial thromboembolism (ATE) when used systemically,[7] and the theoretical risk of thromboembolic events following intravitreal injection of VEGF inhibitor drugs is widely debated.[6,13]

The randomized Comparison of Age-related Macular Degeneration Treatments Trials (CATT) assessed the relative efficacy and safety of ranibizumab and bevacizumab. Although the proportions of patients who experienced ATE were similar between the two drug groups, the proportion of patients who experienced one or more serious systemic adverse events (SAEs) was higher with bevacizumab than with ranibizumab.[14] In contrast, in the IVAN (Inhibition of VEGF in Age-related Choroidal Neovascularization) randomized controlled trial, in which the relative efficacy and safety of ranibizumab and bevacizumab were compared, the frequencies of arterial thrombotic events, heart failure, and at least one systemic SAE were similar between both drug groups.[15]

One of the commonly accepted hypotheses for ATE is related to the class effect of VEGF inhibitors, including ranibizumab, bevacizumab, and aflibercept.[16–20] Suppression of VEGF induces vascular endothelial cell dysfunction, which results in bleeding and thrombosis. There is also evidence that VEGF may modulate the expression of numerous factors involved in hemostasis and thrombolysis.[21–23] Another hypothesis for ATE is attributed to the presence of an Fc region within the structures of anti-VEGF drugs. Intriguingly, in vitro

experiments showed that bevacizumab forms immune complexes (ICs) with VEGF and activates platelets via FcγRIIa, in a similar manner to heparin-induced thrombocytopenia (HIT). In general, heparin inhibits reactions that lead to the clotting of blood and the formation of fibrin clot. HIT is an immune-mediated adverse drug effect and is a syndrome associated with thrombosis[24] in which IgG antibodies form ICs with heparin and platelet factor 4 (PF4) antigen on the platelet surface and activate FcγRIIa. FcγRIIa is a member of the Fc receptor subgroup that recognizes IgGs (FcγR) and is the only FcγR expressed on human platelets. Recently, some thrombotic complications are considered as result from unbalanced FcγRIIa-mediated platelet aggregation.[25] Moreover, FcγRIIa is not activated by individual IgG molecules, but by clustered IgGs, such as those found in ICs.[26]

Ranibizumab, bevacizumab, and aflibercept differ in their structures; bevacizumab and aflibercept have an Fc region, but ranibizumab does not. This raises the important question of whether these drugs have different effects on platelet coagulation. Thus, we investigated whether ranibizumab, bevacizumab, and aflibercept form ICs and activate platelets via FcγRIIa. First, to confirm the formation of a drug–growth factor complex, dynamic light scattering (DLS) methods were employed to investigate possible IC formation. Second, an in vitro platelet aggregation assay was conducted in the presence of each drug and its target molecules to confirm platelet aggregation under more physiological conditions.

Methods

Reagents

Anti-human CD32 (FcγRIIa) antibody (Ab), was obtained from STEMCELL Technologies (Vancouver, BC, Canada), and bevacizumab and ranibizumab from Genentech. Aflibercept was obtained from Regeneron (Tarrytown, NY, USA) and recombinant human VEGF-A165, human VEGF-B167, human placental growth factor (PlGF), and purified human IgG from R&D Systems (Minneapolis, MN, USA). Heparin sodium was obtained from Ajinomoto (Tokyo, Japan) and the fluorescent labeling (IgG labeling reagent) from Molecular Probes (Eugene, OR, USA).

Dynamic Light Scattering

To confirm the formation of a drug–growth factor complex, DLS measurements were performed with a ZetaSizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK) equipped with a He-Ne laser ($\lambda = 633$nm) as the incident beam. All measurements were taken at 25°C and a detection angle of 173°. Growth factors (40 μM) and individual drugs (20 μM) were mixed and incubated for 12 hours at room temperature. Twenty microliters of each sample was loaded into a low-volume cuvette (Zen2112; Malvern Instruments Ltd.). Scattered light intensity was measured to monitor the formation of drug–growth factor complexes, using a constant attenuator setting in the instrument. Cumulative size and size distribution (volume-weighted) histograms were calculated based on the autocorrelation function of the samples, with automated attenuator adjustment and multiple scans for optimal accuracy.

Subjects

Blood samples were obtained from 16 subjects (5 AMD patients and 11 healthy volunteers). The mean age of the patients was 79 years and three were female. Three patients were receiving antiplatelet therapy and two had a history of stroke (cerebral infarction). The three patients without a history of stroke had received several VEGF inhibitor intravitreal injections. The mean age of the normal volunteers was 41 years and four were female.

Platelets

Platelets were collected in a standardized manner as previously described.[27] Blood was stored in sodium citrate (1/10 volume) and platelets were prepared by centrifugation (160*g*, 12 minutes). Platelets were washed twice in buffer (138 mM NaCl, 2.9 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 3.3 mM NaH_2PO_4, 1 mg/mL glucose, pH 7.4) containing 15% acid-citrate dextrose (ACD) and 100 nM prostaglandin I2 (PGI2). Washed platelets were resuspended (200,000/μL) in buffer, and 1 mM $CaCl_2$ and 500 μg/mL fibrinogen were added. Institutional review board approval was obtained from the University of Tokyo. Written informed consent was obtained from all subjects.

Aggregation Study

Platelet aggregability was assessed using an aggregometer (model PA-200; Kowa, Tokyo, Japan) that can detect even small aggregates consisting of only two or three platelets with the following method,[28] which uses a conventional technique based on changes in the light transmission (LT) of the platelet suspension. Briefly, 270 μL washed platelets were maintained at 37°C in a cylindrical glass cuvette and stirred with a magnetic bar at 1000 rpm. A diode laser light beam (width, 40 μm; wavelength, 675 nm) was passed through a limited area of the sample, and the intensity of the light scattered by particles was recorded by a four-channel photodiode array that minimized multiple light-scattering signals. Each of the four photodiodes detected the light scattered from particles in its corresponding observation volume (one photodiode for each observation volume). The flow direction of the stirred particles was diagonal to the line of the four-channel photodiode array, thus the same particle could not pass two or more photodiodes successively. The light signals obtained were digitized with a computer and data were recorded on a two-dimensional plot, one parameter being time (seconds) and the other being total light intensity expressed as arbitrary units (AU). The magnitude of platelet aggregation assessed by this method was expressed as percentage of LT (percent increase in the LT of washed platelets relative to the LT of control buffer). Preliminary experiments performed in our laboratory showed that sensitivity toward platelet aggregation was increased by priming platelets with 2 μM adenosine diphosphate (ADP). Briefly, the aggregation study was conducted under the following conditions: 270 μL washed platelets were maintained at 37°C in a cylindrical glass. One minute after starting the assessment of platelet aggregability, ADP was added to the platelets at a final concentration of 2 μM. After 5 minutes, the VEGF inhibitor drugs (ranibizumab, bevacizumab, or aflibercept) and growth factors (VEGF-A, VEGF-B, or PlGF) were added to the platelets at the final concentration of 240 nM for both drugs and growth factors. Platelet aggregability was assessed for 30 minutes. Significant aggregation was defined as percent LT surpassing 20% at 30 minutes after the assessment had commenced. To further investigate the effects of heparin, samples containing a VEGF inhibitor drug and a growth factor together with heparin (final concentration of 0.1 U/mL) were also assessed. Additionally, where indicated, anti-FcγRIIa mouse antibody was added before measuring aggregation to clarify whether platelet stimulation involves the Fc receptor, VI.3.

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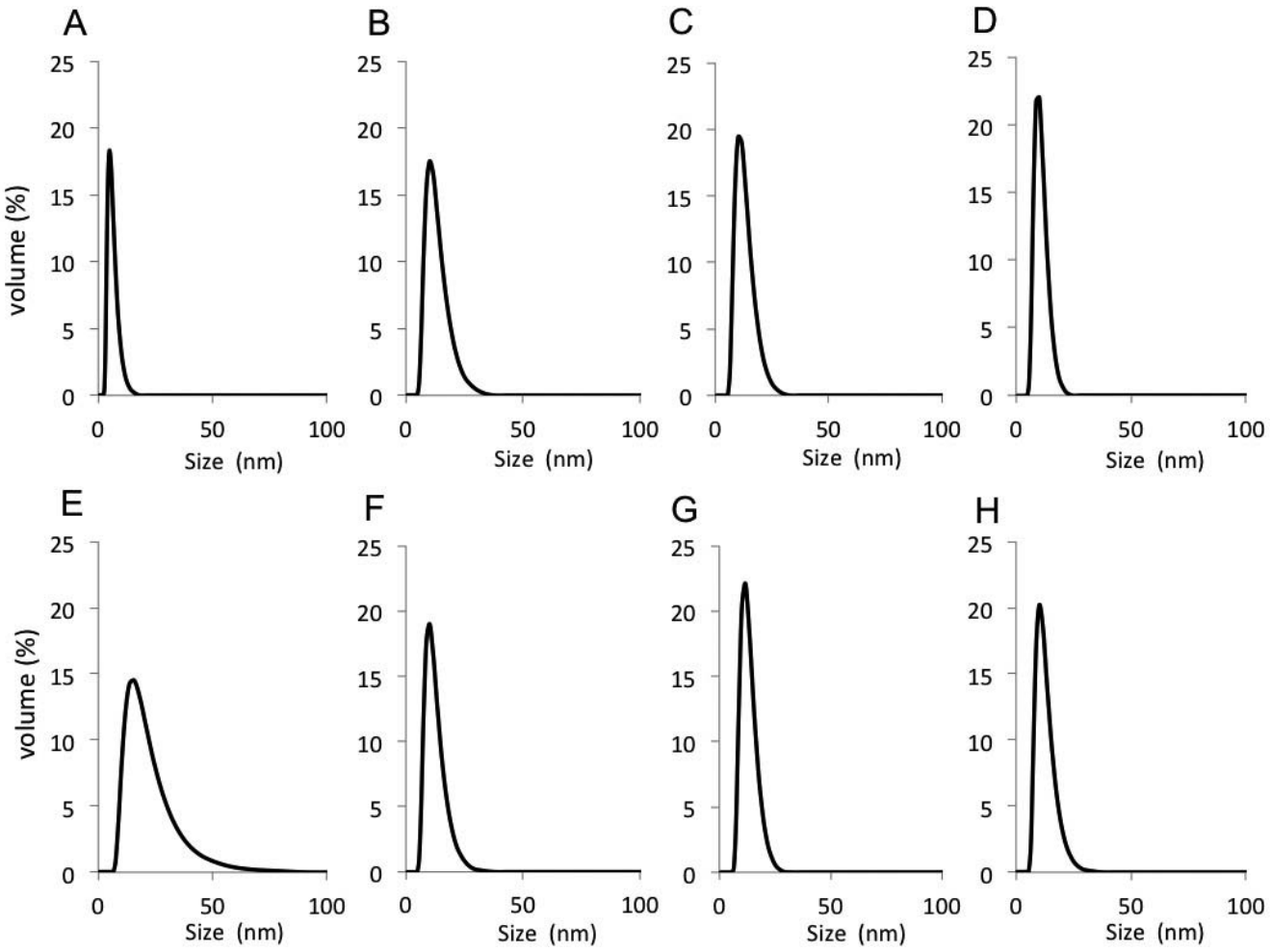

FIGURE 1. The volume-weighted, size distribution histograms of each drug or drug–growth factor complex. Ranibizumab (**A**), bevacizumab (**B**), and aflibercept (**C**) each showed one peak. The mixture of ranibizumab and VEGF-A (**D**) also showed one peak. The mixture of bevacizumab and VEGF-A showed one peak with a relatively gentle slope, indicating a heterogeneous mixture of multimeric bevacizumab–VEGF-A complexes (**E**). The mixture of aflibercept and VEGF-A (**F**), aflibercept and VEGF-B (**G**), and aflibercept and PlGF (**H**) showed only one steep peak.

Giemsa Staining

After the aggregation study, a platelet smear from each sample was stained by Giemsa for 30 minutes.

Fluorescent Labeling

To confirm the presence of the Fc region in the aggregates, human IgG labeling reagent (Molecular Probes), a fluorophore-labeled Fab fragment directed against the Fc portion of an IgG primary antibody, was added into each drug–growth factor mixture before the aggregation study and fluorescence was observed with a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). Platelet aggregates formed by collagen were used as controls.

RESULTS

Dynamic Light Scattering

The size distribution histograms of each drug or drug–growth factor complex are shown in Figures 1A to 1C. Ranibizumab, bevacizumab, and aflibercept each showed one peak. A mixture of ranibizumab and VEGF-A (Fig. 1D) also resulted in one steep peak. In contrast, a mixture of bevacizumab and VEGF-A showed one peak with a relatively gentle slope, indicating a heterogeneous mixture of multimeric bevacizumab–VEGF-A complexes (Fig. 1E). Mixtures of aflibercept and VEGF-A, aflibercept and VEGF-B, and aflibercept and PlGF (Figs. 1F–H) each showed only one steep peak.

Platelet Aggregation With Ranibizumab

All platelet samples showed an increase in LT of less than 20% in the presence of VEGF-A, VEGF-B, or PlGF, and no detectable aggregation was observed. A typical pattern of results obtained with ranibizumab is shown in Figure 2A. All results with ranibizumab were similar to those in Figure 2A. Even when heparin was added, no detectable aggregation was observed (Fig. 3A).

Platelet Aggregation With Bevacizumab

Although no detectable aggregation was observed in the presence of VEGF-B or PlGF, significant aggregation was detected in the presence of VEGF-A in 2 volunteers, and LT increased by 41% and 64%, respectively (Fig. 2B-a). Significant aggregation was observed in five cases (1 patient and 4 volunteers; Fig. 3B) when heparin was added, and LT increased by 41%, 52%, 23%, 21%, and 70% in these subjects (Fig. 2B-b).

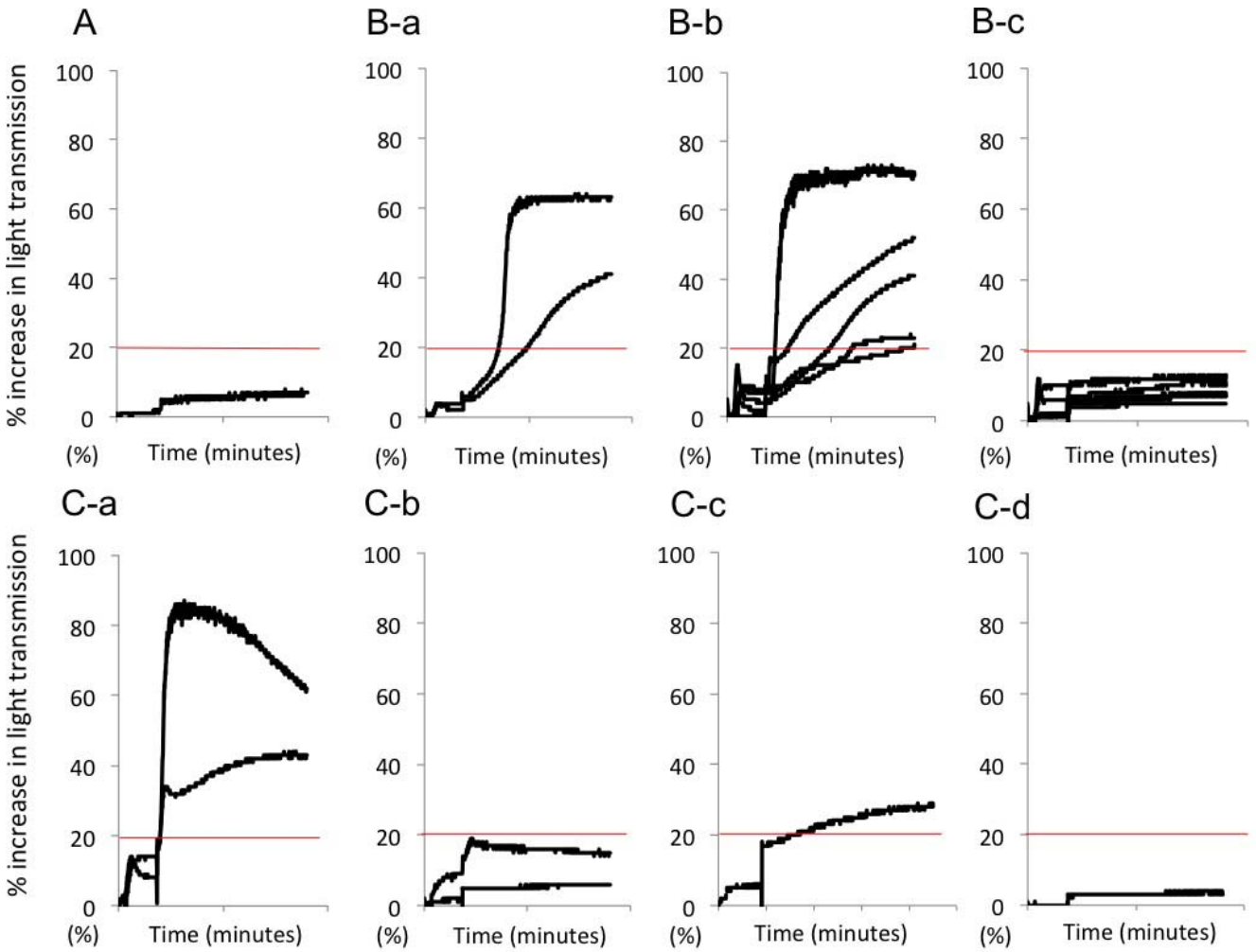

FIGURE 2. Light transmission (LT) of platelet suspension (%). (**A**) No detectable aggregation was observed with ranibizumab. (**B**) Platelet aggregation with bevacizumab. (**a**) Significant aggregation was observed in the presence of VEGF-A in two cases. (**b**) When heparin was added, significant aggregation was observed in five cases in the presence of VEGF-A. (**c**) Aggregation was not observed in any of the five cases when VI.3, an anti-FcγRIIa monoclonal antibody (mAb), was added to the platelets in advance. (**C**) Platelet aggregation with aflibercept. (**a**) Significant aggregation was observed in the presence of VEGF-B with heparin in platelets from two volunteers. (**b**) When VI.3 anti-FcγRIIa mAb was added to platelets in advance, no aggregation was observed in these two cases. (**c**) Significant aggregation was observed in the presence of PlGF with heparin in platelets from one volunteer. (**d**) When VI.3 anti-FcγRIIa mAb was added to platelets in advance, aggregation was not observed in the presence of PlGF with heparin in the platelets. The *red bar* indicates 20% LT. Ab, antibody.

When VI.3 anti-FcγRIIa monoclonal antibody (mAb) was added to the platelets in advance, aggregation was absent in all five cases (Figs. 2B-c, 3C). No aggregation was observed in the absence of growth factors.

Platelet Aggregation With Control IgG

Significant aggregation of platelets from two volunteers was observed in the presence of VEGF-B with heparin (Figs. 2C-a, 3D), and the LT was 43% and 62%. Conversely, aggregation was not observed in these two cases when VI.3 anti-FcγRIIa mAb was added to the platelets in advance (Figs. 2C-a, 3E). Without heparin, aggregation in the presence of VEGF-B was observed in one case only, which was inhibited by VI.3. No detectable aggregation was observed in the presence of VEGF-A. In the presence of PlGF and heparin, significant aggregation was observed in one volunteer (Fig. 2C-c), which was inhibited by VI.3 (Fig. 2C-d). No aggregation was observed in the absence of growth factors.

Platelet Aggregation With Control Igg

No detectable aggregation was observed in the presence of VEGF-A, VEGF-B, or PlGF.

Fluorescent Labeling

When the fluorescent IgG label was added before aggregation, fluorescence was clearly observed in aggregates with both bevacizumab and VEGF-A (Fig. 4D) and with aflibercept and VEGF-B (Fig. 4E). As expected, no fluorescence was observed in platelet aggregates formed by collagen (Fig. 4F).

DISCUSSION

The addition of bevacizumab to chemotherapy is reported to increase the risk of ATE when used systemically.[29] According to the pooled data from five randomized controlled trials which included a total of 1745 patients with metastatic colorectal,

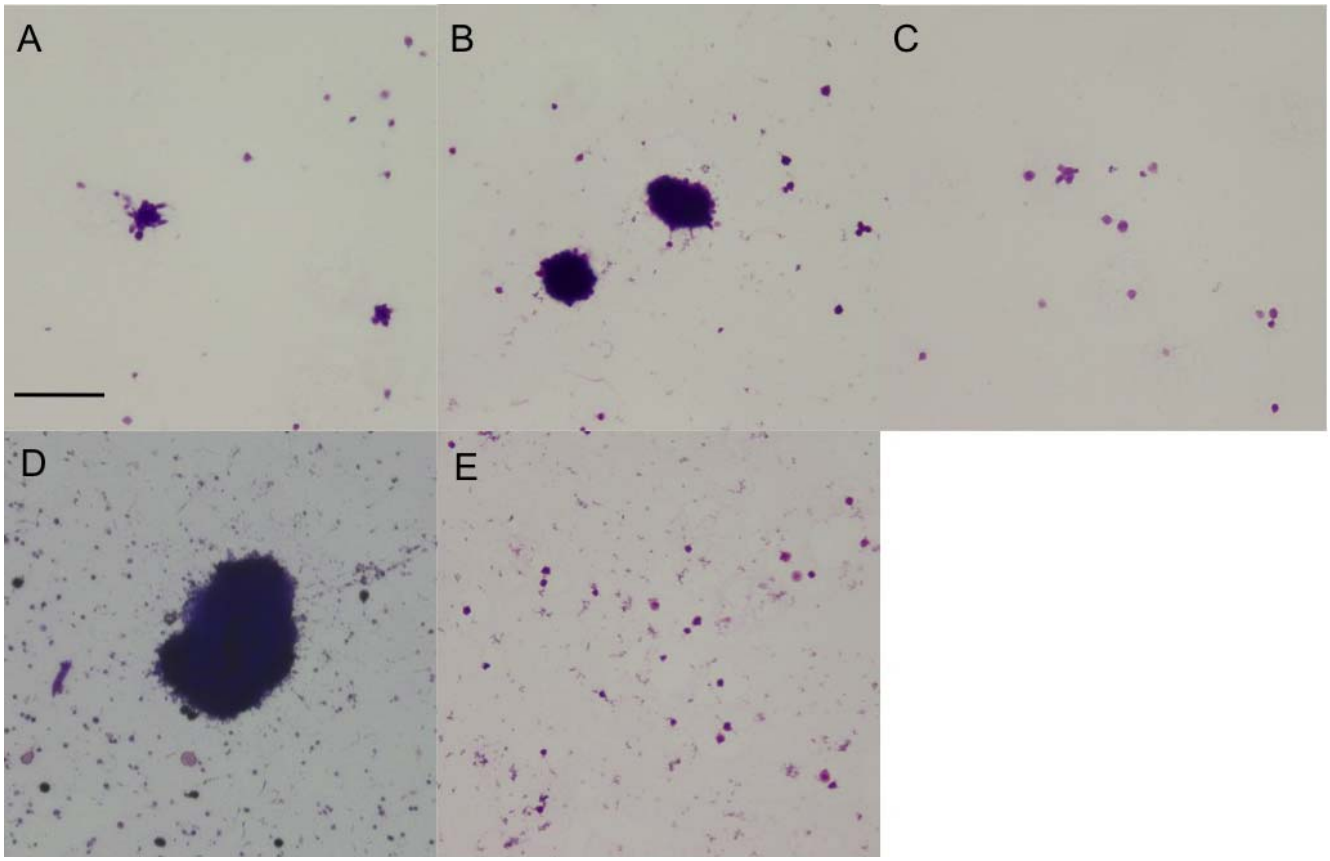

FIGURE 3. Giemsa stain smears. (**A**) No significant aggregation was observed with ranibizumab. (**B**) Platelet aggregation with bevacizumab and VEGF-A. (**C**) No significant aggregation was observed with bevacizumab and VEGF-A in the presence of anti-FcγRIIa mAb. (**D**) Platelet aggregation with aflibercept and VEGF-B. (**E**) No significant aggregation was observed with aflibercept and VEGF-B in the presence of anti-FcγRIIa mAb. *Scale bar*: 50 μm.

breast, or nonsmall-cell lung carcinoma, combined treatment with bevacizumab and chemotherapy, compared with chemotherapy alone, was associated with increased risk for an arterial thrombolic event (Hazard ratio = 2.0, 95% confidence interval = 1.05–3.75; $P = 0.031$).[29] As of November, 2015, aflibercept is approved for metastatic colorectal cancer that is resistant to or had progressed after an oxaliplatin regimen, in combination with 5-fluorouracil, leucovorin, irinotecan (FOLFIRI) in the United States and the European Union. In a recent Phase III study, arterial thromboembolic events were seen in 1.8% in the aflibercept + FOLFIRI arm compared with 0.5% in the placebo + FOLFIRI arm. Venous thromboembolic events were seen in 8.2% of patients receiving aflibercept + FOLFIRI regimen compared with 6.4% receiving placebo + FOLFIRI regimen.[30] Compared with bevacizumab, the risk of thromboembolic event after systemic aflibercept administration has not been thoroughly investigated so far. The theoretical increased risk of such events following intravitreal injection of VEGF inhibitors (ranibizumab, bevacizumab, and aflibercept) is still controversial and widely discussed.[6,13,31,32] Some studies show an association between ATE with the use of intravitreal anti-VEGF drugs, whereas others do not.[6,33]

There are several hypotheses regarding the mechanisms of the increased risk of arterial thrombosis with VEGF inhibitor treatment. In the current analysis, we tested the hypothesis that thrombosis associated with the use of bevacizumab results from IC-mediated activation of platelets.[24] According to this hypothesis, bevacizumab–VEGF–heparin complexes form, and can bind and subsequently activate platelets through FcγRIIa. In the present size distribution histogram by DLS, the mixture of VEGF-A and bevacizumab showed a peak with a gentle slope, indicating that VEGF-A and bevacizumab form multimeric complexes; thus, this observation supports the aforementioned hypothesis. A previous study using bovine retinal microvascular endothelial cell proliferation assays have shown that the potency of bevacizumab toward VEGF-A is six times less than the potency of aflibercept and ranibizumab toward the same growth factor.[34] Despite this bias, bevacizumab formed multimeric complexes, whereas we failed to confirm whether the mixtures of ranibizumab or aflibercept were heterogeneous. Accordingly, under the current conditions in vitro, only VEGF-A and bevacizumab formed a stable complex that was readily detected by DLS.

In the platelet aggregation study, which assessed effects under more physiological conditions, significant aggregation was observed in the presence of VEGF-A and bevacizumab, and VEGF-B and aflibercept, while no detectable aggregation was observed in the presence of VEGF-A, VEGF-B, or PlGF in combination with ranibizumab. When heparin was added, significant aggregation was observed with VEGF-A and bevacizumab, VEGF-B and aflibercept, and PlGF and aflibercept. Notably, VI.3 anti-FcγRIIa mAb inhibited aggregation in all cases. The results of this study supported the hypothesis that VEGF inhibitor–growth factor–heparin complexes may bind and activate platelets through FcγRIIa. In the case of aflibercept, there is a discrepancy between the results of DLS and those of the aggregation study. A possible explanation for this might be that the aflibercept complex was not stable enough to be detected using DLS. Alternatively, aflibercept might form a complex and bind to the FcγRIIa only in the

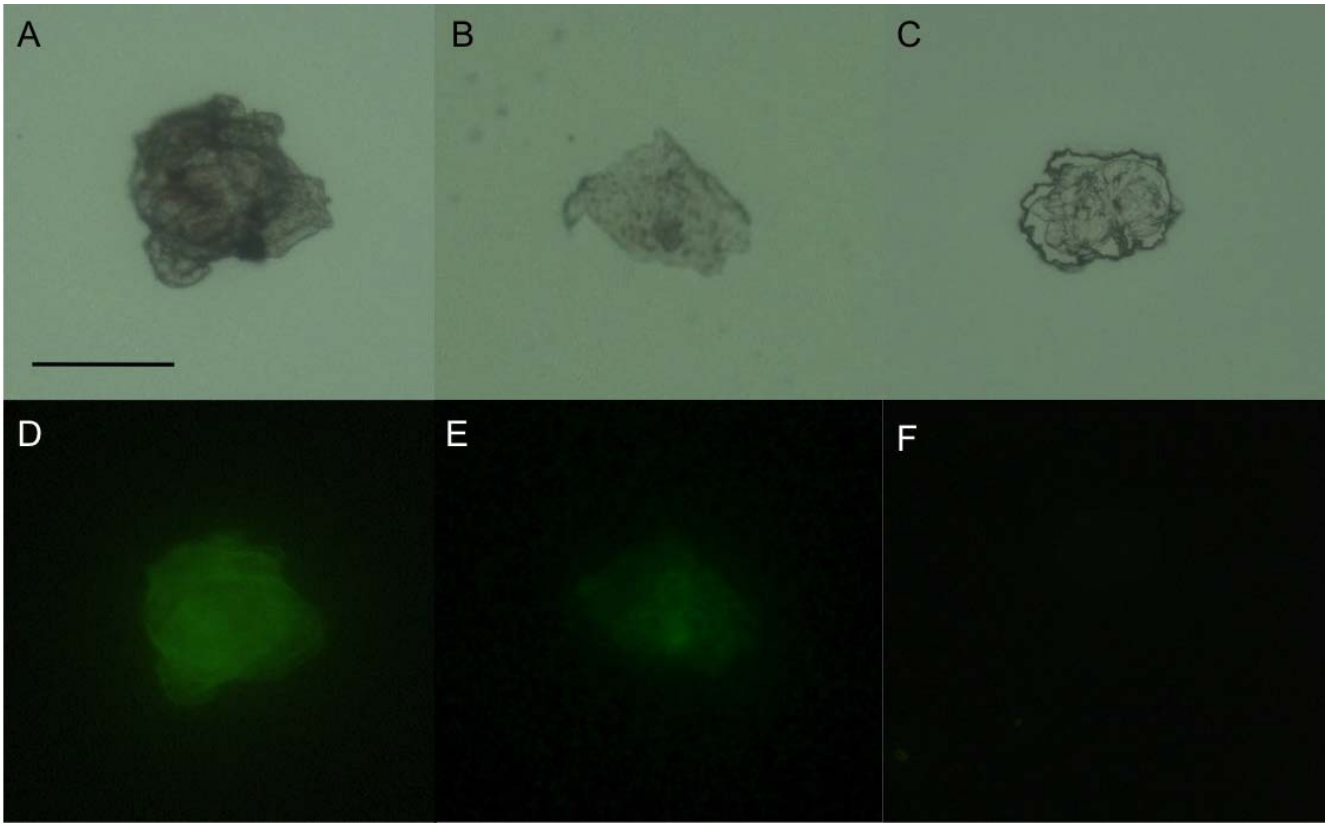

FIGURE 4. Fluorescent labeling. A fluorescent label was added before aggregation. (**A**) Platelet aggregation with bevacizumab and VEGF-A. (**B**) Platelet aggregation with aflibercept and VEGF-B. (**C**) Platelet aggregation stimulated by collagen as the control. (**D**) Fluorescence was clearly observed in the aggregates with bevacizumab and VEGF-A. (**E**) Fluorescence was clearly observed in the aggregates with aflibercept and VEGF-B. (**F**) Platelet aggregates stimulated by collagen showed no fluorescence. *Scale bar*: 50 μm.

presence of platelets by a currently unknown mechanism, although further studies are needed to confirm this. In this study, heparin was dispensable for activation of platelets in some subjects, but aggregation was observed more frequently in the presence of heparin, indicating that heparin has a stimulatory effect on platelets, which leads to the formation of complexes of bevacizumab and VEGF-A, aflibercept and VEGF-B, and aflibercept and PlGF. When a fluorescent label directed against the Fc portion was added before aggregation, fluorescence was clearly observed in the aggregates in the presence of bevacizumab and VEGF-A, and aflibercept and VEGF-B. As expected, platelet aggregates stimulated by collagen showed no fluorescence. These results showed that the aggregates contained both bevacizumab and aflibercept, thus supporting the hypothesis that VEGF inhibitor–growth factor–heparin complexes bind and activate platelets.

Vascular endothelial growth factor plays an important role in endothelial cell function, proliferation, and survival.[16,17] One prevalent hypothesis related to the increased risk of ATE with the use of anti-VEGF drugs is that the inhibition of VEGF signaling results in decreased endothelial cell survival and increased apoptosis due to vascular injury, which leads to disruption of the endothelial cell barrier and exposure of subendothelial von Willebrand factor (vWF) and tissue factor (TF), and subsequent platelet aggregation and thrombus formation.[16,35] There is also evidence to indicate that VEGF may modulate the expression of numerous factors involved in both hemostasis and thrombolysis. Nitric oxygen (NO) and PGI2, both inhibitors of platelet activation, are increased upon endothelial cell stimulation with VEGF.[21–23] Vascular endothelial growth factor inhibition may affect these factors and shift the homeostatic balance in favor of thrombosis. This is the class effect of anti-VEGF drugs, which is not generally considered drug-specific. Further studies are needed to clarify this.

Despite positive results, this study has some limitations. First, it was an in vitro study and did not accurately mimic the events that occur in vivo. In the current platelet aggregation assay, the concentration of each VEGF inhibitor is 240 nM (i.e., 11,520 ng/mL ranibizumab, 35,760 ng/mL bevacizumab, and 27,600 ng/mL aflibercept). In a previous study using rabbits, the maximum concentration of plasma bevacizumab was 2087 ng/mL at 2 weeks after intravitreal injection,[36] and ranibizumab was not detected in the serum after intravitreal injection.[37] In human, systemic exposure (Cmax) after first intravitreal injection of aflibercept and bevacizumab is estimated to be 0.45 nM and 0.76 nM, respectively, which are much higher than that of ranibizumab (0.11 nM).[38] We used higher concentrations of VEGF inhibitors than those used in the aforementioned studies so that we could obtain mechanistically informative results. According to a previous study, the plasma VEGF concentration before intravitreal treatment in AMD patients was 180 to 190 pg/mL, which is much lower than that in the present study. Thus, the experimental condition of this study does not completely mimic the clinical situation; however, this study demonstrated functional difference depending on the Fc portion. Second, the function of heparin was not revealed in this study. However, heparin therapy or heparin flush treatments are often used in hospital patients, specifically in AMD patients who are elderly and susceptible to hospitalization due to various illnesses, thus the mechanism demonstrated in this study is of importance when choosing appropriate therapeutic options. Third, we

examined the platelets of only 16 subjects who are all Japanese. Further studies of a larger series of patients are required to reach more reliable conclusions. Lastly, clinical data on whether there is an increased risk of ATE with the use of bevacizumab and aflibercept compared with ranibizumab are still lacking. This is a very important issue that needs to be addressed in future clinical studies.

Although the role of VEGF in thrombosis is complex and the mechanisms of VEGF inhibitor-associated thromboembolism are not clear, this study showed that bevacizumab and aflibercept might form ICs with growth factors and activate platelets via FcγRIIa, which leads to aggregation.

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