Combination of AIBP, apoA-I, and Aflibercept Overcomes Anti-VEGF Resistance in Neovascular AMD by Inhibiting Arteriolar Choroidal Neovascularization

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Purpouse. Anti-VEGF resistance represents a major unmet clinical need in the management of choroidal neovascularization (CNV). We have previously reported that a combination of AIBP, apoA-I, and an anti-VEGF antibody overcomes anti-VEGF resistance in laser-induced CNV in old mice in prevention experiments. The purpose of this work is to conduct a more clinically relevant study to assess the efficacy of the combination of AIBP, apoA-I, and aflibercept in treatment of anti-VEGF resistance of experimental CNV at different time points after laser photocoagulation.

Methods. To understand the pathobiology of anti-VEGF resistance, we performed comprehensive examinations of the vascular morphology of laser-induced CNV in young mice that are highly responsive to anti-VEGF treatment, and in old mice that are resistant to anti-VEGF therapy by indocyanine green angiography (ICGA), fluorescein angiography (FA), optical coherence tomography (OCT), and Alexa 568 isolecitin labeled choroid flatmounts. We examined the efficacy of the combination therapy of AIBP, apoA-I, and aflibercept intravitreally delivered at 2, 4, and 7 days after laser photocoagulation in the treatment of CNV in old mice.

Results. Laser-induced CNV in young and old mice exhibited cardinal features of capillary and arteriolar CNV, respectively. The combination therapy and the aflibercept monotherapy were equally effective in treating capillary CNV in young mice. In old mice, the combination therapy was effective in treating anti-VEGF resistance by potently inhibiting arteriolar CNV, whereas aflibercept monotherapy was ineffective.

Conclusions. Combination therapy of AIBP, apoA-I, and aflibercept overcomes anti-VEGF resistance in experimental CNV in old mice by inhibiting arteriolar CNV.

Keywords: choroidal neovascularization (CNV), apolipoprotein A-I binding protein (AIBP), anti-VEGF resistance, age-related macular degeneration (AMD), indocyanine green angiography (ICGA)

Age-related macular degeneration (AMD) is a major cause of blindness in the elderly that is rapidly increasing in prevalence with the aging of the population. In the United States, the number of patients with AMD is expected to increase substantially from 11 million to nearly 22 million by 2050, whereas the global prevalence is expected to increase to 288 million by the year 2040. Choroidal neovascularization (CNV or wet AMD), which accounts for 10% to 20% of AMD, is responsible for 80% to 90% of blindness in patients with AMD. Anti-vascular endothelial growth factor (VEGF) therapies that target extracellular VEGF have revolutionized the treatment of CNV. However, up to 50% of patients have suboptimal responses and outcomes to this treatment with evidence of persistent disease activity, such as persistent fluid and unresolved or new hemorrhage. The long-term outcomes can be suboptimal even among responders and can lead to anti-VEGF resistance. For example, 51.5% of patients receiving intravitreal ranibizumab and 67.4% of patients treated with bevacizumab had persistent fluid despite monthly treatment for 2 years (CATT trial). There were 19.7% to 36.6% of patients who had active exudation after 1 year of regular 2.0 mg aflibercept treatments (VIEW 1 and VIEW 2 trials). The mean visual acuity gradually decreased during long-term follow-up with retreatment using a pro re nata (prn) regimen when patients exited from the MARINA or ANCHOR trial (SEVEN-UP study). Different strategies have been tested to overcome this issue, including increasing the frequency of anti-VEGF therapy and switching to different anti-VEGF agents. Various combination therapies have been explored in clinical trials, including targeting platelet-derived growth factor (PDGF; Fovista) and angiopoietin-2 (e.g. nesvacumab and faricimab). However, no major breakthroughs have been reported in combating anti-VEGF resistance. Thus, development of an effective therapy addressing anti-VEGF resistance represents an important unmet clinical need.

CNV is driven by abnormal levels of angiogenesis and inflammation, in which VEGF in mice, we will use VEGF for simplicity hereafter), endothelial cells, and macrophages are critically involved. In particular,
accumulating evidence from both animal models and human patients suggests that macrophages play important roles in the pathogenesis of wet AMD.\(^{11-22}\) Specifically, dysregulation of macrophage cholesterol homeostasis has been implicated,\(^{20,23}\) and hyper-reflective lipid-filled macrophages or microglia have been detected in wet AMD.\(^ {24-27}\) Notably, macrophages have increased density and proliferative activity in response to bevacizumab treatment, suggesting that macrophages play a role in anti-VEGF resistance.\(^ {28}\) These studies prompted us to explore a new treatment strategy for CNV by targeting VEGF, endothelial cells, and macrophages to address the limitations of current anti-VEGF monotherapy.

We and others reported that the secretory apolipoprotein A-I (apoA-I) binding protein (AIBP) enhances cholesterol efflux in endothelial cells and macrophages.\(^ {20-32}\) AIBP binds its partner apoA-I or high-density lipoprotein (HDL) to enhance cholesterol efflux and inhibit lipid raft-anchored VEGFR2 signaling in endothelial cells.\(^ {29}\) By binding to the toll-like receptor 4 (TLR4), AIBP also augments cholesterol efflux from cholesterol-laden, inflamed macrophages/microglia, normalizes plasma lipid rafts, and suppresses inflammation.\(^ {30,31,33,34}\) The unique properties of AIBP that target both endothelial cells and macrophages makes it an ideal drug candidate to work synergistically with anti-VEGF agents to treat CNV. Indeed, we recently found that a combination of AIBP, apoA-I, and an anti-VEGF neutralizing antibody eliminated anti-VEGF resistance and effectively suppressed CNV in mice when applied immediately after laser photocoagulation (i.e. prevention experiment).\(^ {32}\)

To develop an effective therapy that addresses the limitation of current anti-VEGF treatments, it is essential to develop a clinically relevant animal model of CNV that is resistant to anti-VEGF treatment. Multiple pivotal clinical trials have shown that in human patients, advanced age and larger CNV lesions at baseline are associated with worse anti-VEGF treatment outcomes (e.g. ANCHOR, MARINA, and CATT studies).\(^ {8,35-37}\) We found that laser photocoagulation produced larger CNV lesions in aged mice, that were much more resistant to anti-VEGF treatment compared with those in young mice.\(^ {32}\) Furthermore, anti-VEGF resistance in patients with CNV is frequently associated with arteriolar CNV, which is characterized by large-caliber branching arterioles, vascular loops, and anastomotic connections.\(^ {7}\) Current anti-VEGF treatment can lead to vessel abnormalization, arteriolar CNV formation, and anti-VEGF resistance.\(^ {2,38}\) On the other hand, anti-VEGF responders are characterized by having capillary CNV, in which leakage occurs because of VEGF-mediated permeability of leaky capillaries.

In this study, we performed comprehensive examination of laser-induced CNV in young and old mice by fluorescein angiography (FA), indocyanine green angiography (ICGA), spectral-domain optical coherence tomography (SD-OCT), and retinal pigment epithelium (RPE)-choroidal flatmount imaging. We found that laser-induced CNV in young and old mice exhibits cardinal features of capillary and arteriolar CNV, respectively. We then performed a more clinically relevant experiment comparing the efficacy of AIBP/apoA-I/aflibercept combination therapy with the aflibercept monotherapy in the treatment of CNV in young and old mice at different time points after laser photocoagulation. Our data show that the combination therapy but not the aflibercept alone overcomes anti-VEGF resistance by potently inhibiting arteriolar CNV.

**METHODS**

**Mice**

Wild type (WT; C57BL/6j) mice were purchased from Jackson Laboratory. Naxex\(^ {37-38}\) mice (C57BL/6N-A\(^ {m1bhrd}\) Apoa1bptm1a(EUCOMM)Hmgu /BayMmucd, RRID:MMRRC 041520-UCD) were obtained from the Mutant Mouse Resource and Research Center (MMRRC) at University of California at Davis. Old male and female C57BL/6 mice (9–12 months) were ordered from the Comparative Medicine of Baylor College of Medicine and maintained in house until they reached the desired age (12–15 months for females and 16–18 months for males). We have shown previously that laser-induced CNV in both old male and female mice exhibit anti-VEGF resistance.\(^ {32}\) All animal experiments were approved by the Institutional Animal Care and Use Committees (IACUC) at Baylor College of Medicine and were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

**Laser-Induced Choroidal Neovascularization**

Laser photocoagulation was carried out as described previously using the Micron IV retinal imaging system (Phoenix Research Lab, Pleasanton, CA, USA) with the Meridian Meridian Meridian Retinal Imager. Laser photocoagulation was carried out as described previously using the Micron IV retinal imaging system (Phoenix Research Lab, Pleasanton, CA, USA) with the Meridian Meridian Retinal Imager.

**Intravitreal Delivery**

Intravitreal injection in mice was performed with an injection volume of 1.2 μL as previously described.\(^ {39}\) A combination of 2.4 μg AIBP, 4.8 μg apoA-I, and 2 μg aflibercept (combination therapy) or anti-VEGF monotherapy (2 μg aflibercept and 7.2 μg BSA) or control (7.2 μg BSA and 2 μg human IgG1 isotype control [Bio X cell, Lebanon, NH, USA]) were delivered by intravitreal injection at 2, 4, or 7 days after laser photocoagulation. AIBP was expressed as an N-terminal His-tagged protein in E. coli and purified by Ni-NTA chromatography following a standard protocol. ApoA-I was purified from human plasma by size exclusion chromatography as described.\(^ {41}\) Aflibercept was from Regeneron Pharmaceuticals, Inc.

**FA, ICGA, Funduscopy, and SD-OCT**

For all in vivo imaging, mice were anesthetized by intraperitoneal injection of Ketamine (65–100 mg/kg) and Xylazine (10–20 mg/kg) per body weight. Pupils were dilated with 1% Tropicamide (Henry Schein Medical). Funduscopy examinations were performed with MICRON IV from Phoenix.
Research Laboratories (San Ramon, CA, USA). FA, ICGA, and SD-OCT were taken with HRA-OCT device (Spectralis) from Heidelberg Engineering (Heidelberg, Germany). Indocyanine green (Pfaltz & Bauer, 2 mg/kg) and fluorescein sodium (AK-FLUOR, 10 mg/kg) were co-delivered into mice by tail vein injection or intraperitoneal (i.p.) injection. Immediately after injection, the time course of ICGA and FA were recorded. Because immune cells are readily labeled by ICG and accumulate in the laser spots over a period of 1 to 2 weeks following a single injection, which interferes with the imaging of CNV structure, each mouse eye was used to generate one data point for the ICGA timecourse of capillary and arteriolar CNV formation. OCT sessions were performed at 55 degrees field-of-view using the high-resolution mode (signal quality ≥ 35 dB). The focus depth was 10 to 20 D for combined OCT and ICGA. FA, ICGA, and SD-OCT data were exported as 8-bit grayscale image files. The degree of signal quality ≥ 35 dB). The focus depth was 10 to 20 D for combined OCT and ICGA. FA, ICGA, and SD-OCT data were exported as 8-bit grayscale image files. The degree of vascular permeability of each CNV lesion was quantified as the percentage increase in the area of fluorescence between the early and late phases of FA as described. The retinal thickness in CNV lesion areas was measured from nerve fiber layer to RPE. Three to four measurements from different locations from each CNV lesion were taken.

Statistics

Statistics were calculated by the χ² test in Tables. For all figures, data were tested for normality using the Shapiro-Wilk normality test. Because one or more conditions failed the normality test in each data set, a Mann-Whitney test for two groups or Kruskal-Wallis test with Dunn’s post hoc analysis for multiple groups was used for statistical comparison. The significance levels were marked by asterisks (*): *: P < 0.05; **: P < 0.01; ***: P < 0.001; and ****: P < 0.0001. Bars represent mean ± SD in all figures. Statistical significance (P value) was indicated in the figures and figure legends. Statistical analysis was performed with OriginPro or GraphPad Prism.

RESULTS

Characterization of CNV Lesion Types in Young and Old Mice by ICGA

Neovascular AMD in patients and animal models is typically examined by FA to reveal leakage patterns. However, because FA does not show the vasculature morphology of CNV, we used ICGA, which allows penetration through the RPE due to the infrared spectrum of ICG, to characterize the lesion types of laser-induced CNV. Laser-induced CNV in young (7 to 9-week-old mice) mice predominantly exhibited capillary CNV (91.4%) as assessed by ICGA (Fig. 1A, Table 1). In contrast, 87.5% of CNV in old mice (18-month-old male) exhibited capillary CNV (91.4%) as assessed by ICGA (Fig. 1A, Table 1). In contrast, 87.5% of CNV in old mice (18-month-old male) mice exhibited capillary CNV (91.4%) as assessed by ICGA (Fig. 1A, Table 1).

| Table 1. Distribution of Capillary and Arteriolar CNV in Young Versus Old Mice Based on ICGA |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Capillary       | Arteriolar      |                 |                 |                 |                 |                 |                 |
| Young           | 53 (91.4%)      | 5 (8.6%)        |                 |                 |                 |                 |                 |                 |
| Old             | 8 (12.5%)       | 56 (87.5%)      |                 |                 |                 |                 |                 |                 |

Percentage in brackets represents row percentage; χ² (1, N = 122) = 75.7, P < 0.001.

Characterization of CNV Lesion Types in Young and Old Mice by FA

We examined the CNV in young and old mice by time course FA. The fluorescein dye filled in the arteriolar CNV in old mice more rapidly than the capillary CNV in young mice in the early phase FA (Fig. 3A). Old mice also exhibited profound leakage in the late phase FA (see Fig. 3A). To quantitatively compare the vascular permeability of CNV between young and old mice, we measured the change of fluorescent area between the early and late phases of FA. Arteriolar CNV in old mice is associated with 2.3 times increased leakage (Fig. 3B, P < 0.0001). The arteriolar CNV in old mice is likely to produce increased leakage as a result of high rates of blood flow and poorly formed tight junctions at arteriovenous anastomotic loops.

Characterization of CNV Lesion Types in Young and Old Mice by SD-OCT

Cross-sectional OCT scans of capillary CNV in young mice and arteriolar CNV in old mice showed very different features (Fig. 4A). Compared with capillary CNV, arteriolar CNV exhibited much larger subretinal fibro-vascular CNV membrane (dashed red line) with multiple compartment subretinal fluid (red arrows), consistent with the extensive leakage seen in FA (see Fig. 3). Retinal thickness in the lesion area in old mice was significantly increased (40% increase, P < 0.0001; Fig. 4B). In contrast, capillary CNV exhibited no subretinal fluid. RPE, outer nuclear layer (ONL), and outer plexiform layer (OPL) were disrupted in both CNV types.
FIGURE 1. Vascular morphology of laser-induced CNV in young and old mice. (A) Early and late phase ICGA in laser-induced CNV in young mice. Magnified images of the lesion (white arrows) in the left were shown on the right. (B) Early and late phase ICGA in laser-induced CNV in old mice. Magnified images of the lesion in the left were shown on the right. (C) ICGA and the corresponding Alexa 568 isolectin labeled arteriolar CNV. The middle panel shows the magnified image of the lesion in the left (red dashed square). Red arrows indicate a large caliber feeder vessel. (D) Representative images of CNV lesions labeled by Alexa 568 isolectin on RPE/choroid flatmounts in young and old mice. Arrowheads and arrows indicate branching arterioles and vascular loops in old mice, respectively. Scale bar = 40 μm. (E) Quantitative results of normalized CNV area in young and old mice. CNV areas were measured from Alexa 568 isolectin labeled RPE/choroid flatmounts. N = 32 and 40 laser spots in young and old mice, respectively. Bars represent mean ± SD. ****, P < 0.0001.

Role of AIBP in Arteriolar CNV Formation

We determined the role of AIBP in the formation of capillary versus arteriolar CNV by a loss-of-function approach using the Naxe<sup>−/−</sup> (Naxe encoding AIBP) mice. Compared with young WT mice, young Naxe<sup>−/−</sup> mice showed increased arteriolar CNV formation (8.8%) in young WT versus 32.5% in young Naxe<sup>−/−</sup> mice; Fig. 5A). Nevertheless, young Naxe<sup>−/−</sup> mice are still dominated by capillary CNV (67.5%) compared with only 12.5% capillary CNV in old WT mice. Our data
suggest that AIBP does not play a major role in arteriolar CNV formation. Laser-induced CNV in young Natx2−/− mice showed increased CNV area and leakage compared with young WT mice, but reduced CNV area and leakage compared with old WT mice (see Figs. 5B, 5C). In conjunction with previous studies,29,32,48 our data suggest that AIBP accelerates both capillary and arteriolar angiogenesis under physiological and pathological conditions.

**Combination Therapy and Anti-VEGF Monotherapy are Equally Effective in the Treatment of Capillary CNV in Young Mice**

We compared the efficacy of aflibercept with the combination of AIBP/apoA-I and aflibercept in the treatment of CNV in 6 to 9-week-old young mice. We previously reported that a combination of 2.4 μg AIBP and 10 μg apoA-I conferred
maximal inhibition of CNV in mice. Subsequently, we found that maximal inhibition could be achieved using 2.4 μg AIBP and 4.8 μg apoA-I (Supplementary Fig. S1). The clinical dose of aflibercept is 2 mg/human eye, and human vitreous volume of 4.6 to 5.0 mL is approximately 1000 times that of the mouse (5 μL). Thus, we used 2 μg (i.e. 2 mg/1000) aflibercept in this study, a level previously described to be optimal in mice. A combination of 2.4 μg AIBP, 4.8 μg apoA-I, and 2 μg aflibercept or 2 μg aflibercept alone was administered in 6 to 9-week-old mice at day 2 after laser photocoagulation. Seven days after the laser, CNV leakage was assessed by FA, whereas the CNV area was visualized by Alexa-568 isoelectin labeled RPE-choroid flatmounts. The combination treatment was equally effective as the aflibercept monotherapy in reducing CNV leakage (23% reduction) and in reducing the CNV area (approximately 45% reduction) of lesions in young mice that were dominated by capillary CNV (Figs. 6A–D).

Combination Therapy Overcomes Anti-VEGF Resistance by Suppressing Arteriolar CNV

We have shown previously that a combination of AIBP/apoA-I and an anti-VEGF antibody delivered immediately after laser photocoagulation (i.e. prevention) overcame anti-VEGF resistance in old mice. In this experiment, we examined the efficacy of a combination of 2.4 μg AIBP, 4.8 μg apoA-I, and 2 μg aflibercept intravitreally delivered at 2 and 4 days after laser photocoagulation in the treatment of CNV in old mice. At both treatment time points, the AIBP/apoA-I/aflibercept combination therapy significantly inhibited arteriolar CNV formation (Figs. 7A, 7B, white dashed line). These lesions were converted into a mixed-type CNV (see Figs. 7A, 7B, green dashed line; i.e. smaller CNV size with less prominent arteriolar CNV features [large-caliber vessels with branching arterioles, vascular loops, etc.]) and contain capillary CNV (Table 2). Combination therapy significantly reduced both the CNV leakage (42%, $P < 0.001$ and 59%, $P < 0.0001$ reduction for P2 and P4 treatment, respectively; Figs. 7C–F). In sharp contrast, aflibercept monotherapy had no effect in inhibiting arteriolar CNV (see Figs. 7A, 7B, yellow dashed line; Tables 2, 3) or reducing CNV leakage or area (Figs. 7C–F). Because arteriolar CNV was not completely formed until day 7 after laser photocoagulation (see Fig. 2), we performed combination therapy at day 7 after laser treatment in old mice and analyzed at day 10. Combination therapy significantly reduced the number of arteriolar CNV (Table 4) and significantly reduced the CNV leakage (38.5% reduction, $P < 0.0001$; Fig. 7G). However, combination therapy did not significantly reduce the CNV size (Fig. 7H). We did not include aflibercept at P7 treatment because aflibercept showed no benefit even at P2 and P4 treatment. Because the role of aflibercept in reducing VEGF-dependent leakage from capillary CNV is well established (see Fig. 6C), the most parsimonious explanation of the persistent leakage despite aflibercept treatment is that arteriolar CNV causes anti-VEGF resistance (see Figs. 7C, 7E). This experiment suggests that AIBP/apoA-I/aflibercept combination overcomes anti-VEGF resistance by inhibiting arteriolar CNV.

DISCUSSION

The most important finding of this study is that combination therapy of AIBP, apoA-I, and aflibercept overcomes anti-VEGF resistance to aflibercept in experimental CNV in old mice by robustly inhibiting arteriolar CNV. By performing comprehensive in vivo imaging and choroid flatmount analysis, we show that the combination therapy significantly reduces both CNV leakage and size whereas aflibercept monotherapy is ineffective when the treatment was performed at either P2 or P4 after laser photocoagulation. When the treatment was performed at P7, combination therapy significantly reduced the CNV leakage, but not the CNV size. One possible explanation is that it requires longer treatment duration to cause the regression of arteriolar CNV with a network of large caliber vessels. This is difficult to achieve using the short-term laser-induced CNV mouse model, not to mention the short half-time and residence time of
FIGURE 5. Comparison of CNV features in young WT, old WT, and young Naxe<sup>−/−</sup> mice. (A) Distribution of capillary and arteriolar CNV in young WT, old WT, and young Naxe<sup>−/−</sup> mice based on ICGA. Numbers inside the column indicate the number of laser spots. Statistical analysis was calculated by the χ<sup>2</sup> test. (B) The percentage increase of fluorescent area in each CNV lesion between the early and late phases of FA in young WT, old WT, and young Naxe<sup>−/−</sup> mice. N = 50 (young WT), 56 (old WT), and 40 (young Naxe<sup>−/−</sup>) laser spots. (C) Quantitative results of normalized CNV area in young WT, old WT, and young Naxe<sup>−/−</sup> mice. CNV areas were measured from Alexa 568 isolecitin labeled RPE/choroid flatmounts. N = 32 (young WT), 40 (old WT), and 34 (young Naxe<sup>−/−</sup>) laser spots. Young WT (6–9 weeks), old WT (12 to 15-month-old female) mice, young Naxe<sup>−/−</sup> mice (8–12 weeks) were subjected to laser photocoagulation and analyzed at day 7 after laser treatment. Statistical analysis in B and C was performed by Kruskal-Wallis test with Dunn’s post hoc analysis. Bars represent mean ± SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

therapeutics in mouse eyes.<sup>50</sup> This issue will be better addressed using a recently developed rabbit CNV model that is resistant to the anti-VEGF treatment.<sup>54</sup> The much larger eye size, longer half-time and residence time of therapeutics, and the possibility of repeated intravitreal injection (as in the case of human eyes) makes the rabbit model ideal to test the
therapeutic efficacy of the combination therapy as well as for pharmacokinetic studies. Future research will answer these important questions in preparation for an Investigational New Drug (IND) application. Nevertheless, by converting the arteriolar CNV from active exudation to a more quiescent stage, combination therapy offers significant therapeutic advantage in comparison to aflibercept alone.

Because the underlying pathobiology of anti-VEGF resistance is largely unknown, we compared the CNV phenotype of laser-induced CNV in young and old mice by comprehensive in vivo imaging (i.e. ICGA, FA, and OCT). We found that young mice predominantly develop capillary type CNV whereas old mice develop arteriolar CNV, consistent with previous findings based on choroid flatmount imaging of CNV vessel types.7 The vascular morphology of CNV can be readily detected by ICGA and confirmed by Alexa 568 isolectin labeled RPE-choroid flatmounts. To our knowledge, this is the first time ICGA has been applied to examine vascular morphology of CNV in animal models. FA shows that capillary CNV in young mice typically exhibit small lesions, well-demarcated borders, and mild fluorescein leakage while arteriolar CNV in old mice show large, confluent CNV with profound active fluorescein leakage. These features are similar to those observed by ICGA in patients with neovascular AMD.7 Although the mechanism for arteriolar CNV formation is unknown, our hypothesis is that it shares features with arteriogenesis, whereas capillary CNV formation is similar to angiogenesis, in which new capillary blood vessels sprout from a preexisting blood vessel. Although angiogenesis is highly VEGF dependent, arteriogenesis is not VEGF dependent.55–57 In support of this hypothesis, we found that capillary CNV in young mice is highly responsive to aflibercept treatment, whereas arteriolar CNV in old mice is completely resistant to aflibercept treatment. These findings are consistent with clinical findings that anti-VEGF resistance in patients with CNV is frequently associated with arteriolar CNV while anti-VEGF responders are associated with capillary CNV.7 Furthermore, recurrent anti-VEGF treatment can lead to vessel abnormalization and arteriolar CNV formation, which then can lead to anti-VEGF resistance.9,38 These studies provide clinical relevance of using experimental CNV in old animals to model anti-VEGF resistance.

It is well established that monocytes and macrophages play a pivotal role in arteriogenesis by releasing growth factors, proteases, and chemokines, hence mediating cell
FIGURE 7. Comparison between aflibercept and combination therapy (AIBP, apoA-I, and aflibercept) in suppressing laser-induced CNV in old mice. (A) Representative images of FA (early and late phase) and ICGA of CNV lesions after treatments. (B) Representative images of Alexa 568 isoelectin labeled RPE/choroid flatmounts after treatments. (C, E, G) Quantitative results of the percentage increase of fluorescent area in each CNV lesion between the early and late phases of FA in A. N = 40 (control), 34 (aflibercept), and 34 (combination) laser spots in C. N = 33 (control), 35 (aflibercept), and 41 (combination) laser spots in E. N = 44 laser spots for both control and combination.
treatment in G. (D, F, H) Quantitative results of normalized CNV area. N = 59 (control), 34 (aflibercept), and 57 (combination) laser spots in D. N = 56 (control), 34 (aflibercept), and 38 (combination) laser spots in F. N = 42 (control) and 40 (combination) laser spots in H. Old female mice (12–15 months) were treated at day 2 (A through D), day 4 (E and F), and day 7 (G and H) after laser photocoagulation and were analyzed at day 7 (A through F) and day 10 (G and H). Statistical analysis (C through F) was performed by Kruskal-Wallis test with Dunn’s post hoc analysis. Bars represent mean ± SD. NS, *P > 0.05; †, P > 0.05; ‡, P < 0.05; ***P < 0.0001. White and green dashed line indicates arteriolar CNV in control and aflibercept treated mice. Green dashed line indicates mixed type CNV in A and B.

### Table 2. CNV Vessel Type Quantification in Old Mice After Different Treatments Based on Isolectin-B4 Staining (P2 Treatment)

| Arteriolar CNV | Mixed CNV | P Value |
|---------------|-----------|---------|
| Control       | 49 (84.5%)| 9 (15.5%)| 0.375 |
| Aflibercept   | 27 (77.1%)| 8 (22.9%)|         |
| AIBP + apoA-I | 25 (44.6%)| 31 (55.4%)| < 0.0001|

Arteriolar CNV refers to CNVs with prominent branching arterioles and vascular loops. Mixed CNV refers to smaller size CNV with less prominent arteriolar CNV features and that contains capillary CNV. See Figures 7A and 7B CNV type in white and yellow dashed line (arteriolar CNV) and green dashed line (mixed CNV) as examples. Statistics were calculated by the χ² test. Percentage in brackets represents row percentage.

### Table 3. CNV Vessel Type Quantification in Old Mice After Different Treatments Based on Isolectin-B4 Staining (P4 Treatment)

| Arteriolar CNV | Mixed CNV | P Value |
|---------------|-----------|---------|
| Control       | 23 (74.2%)| 8 (25.8%)| 0.993 |
| Aflibercept   | 26 (74.3%)| 9 (25.7%)|         |
| AIBP + apoA-I | 15 (38.5%)| 24 (61.5%)| 0.00287|

Statistics were calculated by the χ² test. Percentage in brackets represents row percentage.

### Table 4. CNV Vessel Type Quantification in Old Mice After Different Treatments Based on Isolectin-B4 Staining (P7 Treatment)

| Arteriolar CNV | Mixed CNV | P Value |
|---------------|-----------|---------|
| Control       | 51 (75.6%)| 10 (24.4%)| 0.0413 |
| AIBP + apoA-I | 21 (53.8%)| 18 (46.1%)|         |

Statistics were calculated by the χ² test. Percentage in brackets represents row percentage.

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