GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Targeting Platelet-Derived Growth Factor Receptor β^+ Scaffold Formation Inhibits Choroidal Neovascularization

Karin Strittmatter, Hayley Pomeroy, and Alexander G. Marneros

From the Cutaneous Biology Research Center, Massachusetts General Hospital, Charlestown; and the Department of Dermatology, Harvard Medical School, Boston, Massachusetts

Accepted for publication
February 22, 2016.

Address correspondence to
Alexander G. Marneros, M.D.,
Ph.D., Cutaneous Biology
Research Center, Massachusetts
General Hospital, CNY-149,
Rm 3.216, 13th St., Charles-
town, MA 02129. E-mail:
amarneros@mgh.harvard.edu.

Neovascular age-related macular degeneration is among the most common causes of irreversible blindness and manifests with choroidal neovascularization (CNV). Anti-vascular endothelial growth factor-A therapies are only partially effective and their chronic administration may impair functions of the choriocapillaris and retina. Thus, novel therapeutic targets are needed urgently. We have observed in a laser-induced model of CNV that a platelet-derived growth factor receptor β positive (PDGFR β^+) scaffold is formed before infiltration of neovessels into this scaffold to form CNV lesions, and that this scaffold limits the extent of neovascularization. Based on these observations we hypothesized that ablation of proliferating PDGFR β^+ cells to prevent the formation of this scaffold might inhibit CNV growth and present a novel therapeutic approach for neovascular age-related macular degeneration. To test this hypothesis we targeted proliferating PDGFR β^+ cells through independent distinct approaches after laser injury: i) by using an inducible genetic model to inhibit specifically proliferating PDGFR β^+ cells, ii) by treating mice with a neutralizing anti-PDGFR β antibody, iii) by administering an anti-PDGF-AB/BB aptamer, and iv) by using small chemical inhibitor approaches. The results show that therapeutic targeting of proliferating PDGFR β^+ cells potently inhibits the formation of the pericyte-like scaffold, with concomitant attenuation of CNV. Moreover, we show that early inhibition of PDGFR β^+ cell proliferation before neovessel formation is sufficient to inhibit scaffold formation and neovascularization. (*Am J Pathol* 2016, 186: 1890–1899; <http://dx.doi.org/10.1016/j.ajpath.2016.02.018>)

Choroidal neovascularization (CNV) occurs in neovascular age-related macular degeneration (AMD) and impairs visual function through abnormal neovessel formation originating from the choroidal vasculature. Anti-vascular endothelial growth factor (VEGF)-A therapies can inhibit CNV and abnormal vascular permeability of these pathologic choroidal neovessels. However, this treatment is not effective in all patients with neovascular AMD.^{1,2} Moreover, long-term anti-VEGF-A therapies may not prevent disease progression even in those patients who showed an initial beneficial therapeutic response, and may lead to detrimental effects on retinal function.³ Thus, novel therapeutic strategies are needed to improve visual outcomes in patients with neovascular AMD.

Here, we assessed the role of pericytes in laser injury-induced CNV lesions, an acute experimental model of

neovascular AMD.⁴ In this model, we found that activated macrophages infiltrate the site of laser injury, stimulate adjacent retinal pigment epithelium (RPE) cells, and promote the migration and proliferation of pericyte-like cells into the site of CNV formation that express the pericyte markers smooth muscle actin (SMA) and platelet-derived growth factor receptor β (PDGFR β).⁴ These pericyte-like cells are not derived from transdifferentiating RPE cells.⁴ Importantly, these pericyte-like cells form a scaffold before the infiltration of CD31⁺ endothelial cells into the site of laser injury and before the formation of neovessels. Only after this SMA⁺PDGFR β^+ pericyte-like scaffold has

Supported by a grant from Ophthotech Corporation.

Disclosures: Fovista was obtained from Ophthotech, which is currently in clinical trials.

formed do endothelial cells migrate along this scaffold and form new blood vessels to establish a CNV lesion. Notably, these pathologic neovessels do not extend beyond the area of this scaffold, and thus the size of CNV lesions is controlled by the extent of the pericyte proliferation and migration.

Although the laser-induced model of neovascular AMD has been used widely to assess inhibition of neovascularization in response to various treatments, the role of the pericyte-like scaffold for CNV lesion formation has not been assessed, and the effects of inhibiting PDGFR β signaling on the formation of this scaffold and of subsequent neovessels in forming CNV lesions has not been determined. Thus, it remains unknown if pharmacologic inhibition specifically of proliferating PDGFR β^+ cells is effective in inhibiting the scaffold and neovessel formation in CNV lesions and whether this approach would be similarly effective in inhibiting CNV as the current standard anti-VEGF-A therapy for neovascular AMD.

Here, we hypothesized that blocking the formation of this pericyte-like scaffold through either genetic or pharmacologic approaches would be likely to inhibit CNV lesion formation and the extent of the neovessels in these lesions. To test this hypothesis we specifically ablated proliferating PDGFR β^+ cells in PDGFRB—thymidine kinase (TK) mice (expressing a viral TK driven by the *Pdgfrb* promoter) that were treated with gancyclovir (GCV) after laser injury, which induces a DNA synthesis arrest only in proliferating PDGFR β^+ cells. We observed a potent attenuation of the SMA $^+$ PDGFR β^+ scaffold and CNV lesion formation in these mice. These observations suggest that therapeutic inhibition of PDGFR β^+ signaling may reduce CNV formation.

Next, we used distinct and independent experimental approaches to inhibit PDGFR β signaling pharmacologically and to provide evidence for an important role of PDGFR β^+ pericyte-like cells for CNV lesion formation. These approaches included small-chemical inhibitor approaches, aptamer approaches, as well as anti-PDGFR β —neutralizing antibodies to block PDGFR β —signaling *in vivo* after laser injury. All of these genetic and pharmacologic experimental approaches consistently showed that targeting PDGFR β signaling potently inhibits laser-induced CNV lesion formation.

Materials and Methods

Inducible Ablation of Proliferating PDGFR β^+ Cells in a Genetic Mouse Model

To ablate proliferating PDGFR β —expressing cells selectively and in an inducible manner, we used transgenic mice that express a PDGFRB-TK transgene. Expression of a viral TK by PDGFR β^+ —proliferating cells renders these cells sensitive to ablation after treatment with GCV.⁵ Thus, treating PDGFRB-TK mice with GCV selectively ablates proliferating PDGFR β^+ cells in an inducible manner. We observed

efficient ablation of PDGFR β^+ cells in laser-induced CNV lesions with daily i.p. administration of GCV at a concentration of 50 mg/kg body weight (BW). GCV treatment was started on the day of laser treatment (day 0) and administered daily until day 7. Additional experimental mouse groups were treated with GCV only on days 0 to 3 or on days 4 to 6 after laser treatment to assess whether inhibition of the proliferation of PDGFR β^+ cells during the early phase of CNV lesion formation (before the angiogenic phase) is sufficient to form the scaffold and neovascularization.

Experimental Treatments

Groups of 8-week-old male C57bl/6j mice ($n = 10$ mice/group) were treated with either phosphate-buffered saline (as vehicle control), fovista (E10030), tivozanib, aflibercept, or aflibercept in combination with fovista. Phosphate-buffered saline (control group) was injected i.p. daily. Fovista (in phosphate-buffered saline) was injected i.p. at a concentration of 50 mg/kg BW/day (obtained from Ophthotech Corp., New York, NY). Aflibercept was injected at a concentration of 12.5 mg/kg BW i.p. on day 0 (day of laser treatment) and on day 3 after laser treatment. Tivozanib was administered daily through oral lavage at a concentration of 1 mg/kg BW, as described previously.^{6,7} The treatment group that received the combination of aflibercept and fovista received 12.5 mg/kg BW aflibercept i.p. on days 0 and 3, and 50 mg/kg BW fovista i.p. daily.

In addition, we treated experimental mouse groups with the neutralizing anti-PDGFR β antibody APB5 and control mice with isotype-matched IgG antibodies (50 μ g/mouse i.p. on days 0, 2, 4, and 6) (eBioscience, San Diego, CA). This treatment has been shown previously to be effective in inhibiting pericytes during tumor angiogenesis in mouse models.⁵

Experimental CNV Model, Immunolabeling Procedures, and Statistical Analysis

Eyes of age- and sex-matched mice were exposed to laser photocoagulation for induction of experimental CNV after eyes had been dilated with 1% tropicamide. Laser photocoagulation was performed using a 532-nm laser (Visulas 532S; Carl Zeiss Meditec, Dublin, Ireland). Lesions were induced using a power of 120 mW, a spot size of 100 μ m, and a duration of 100 ms. Eyes were fixed and examined at 7 days after laser treatment. The size of CNV lesions was measured in choroidal flat mounts. The eyes were enucleated and fixed in 4% paraformaldehyde overnight in 4°C. The anterior segment, the lens, and the retina were removed, and the RPE/choroid tissue (posterior eye) was treated with 0.5% Triton X-100 in 5% blocking buffer (with blocking serum in which the secondary antibody was raised) and subsequently used for immunolabeling. The following antibodies were used for whole-mount immunolabeling of

the RPE/choroid tissue: i) anti-CD31 antibodies (rat anti-mouse CD31: MEC13.3; BD PharMingen, San Jose, CA) to detect blood vessels (Alexa Fluor-488 secondary antibody), ii) anti-SMA antibodies (mouse monoclonal SMA-Cy3 conjugate: clone 1A4; Sigma, St. Louis, MO), iii) anti-neural/glial antigen 2 (NG2) antibodies (rabbit anti-mouse NG2: AB5320; Millipore, Billerica, MA), or iv) anti-PDGFR β antibodies (rat anti-mouse PDGFR β antibody: clone APB5; eBioscience). SMA immunolabeling detected RPE activation and the formation of a fibrovascular scaffold consisting of SMA⁺PDGFR β ⁺ pericyte-like cells and CD31⁺ endothelial cells. In addition, antibodies against F4/80 (rat anti-mouse F4/80-Alexa647 conjugate: clone C1:A3-1; BioLegend, San Diego, CA) were used to detect macrophage infiltration into CNV lesions. Choroidal flat mounts were analyzed by epifluorescence microscopy using a Zeiss microscope (Carl Zeiss Microscopy, Jena, Germany) or with a Nikon confocal microscope (Nikon Instruments, Inc., Melville, NY). Images were obtained with a 20 $\times$ objective. CNV lesions were measured using Zeiss Axio-Vision software version 4.8.2 (Carl Zeiss Microscopy). The average CNV size was determined for each mouse, and differences between mouse groups were assessed by a two-sided nonpaired Student's *t*-test. *P* < 0.05 was considered statistically significant. Exclusion criteria for CNV lesions were the formation of a retinal plug and abnormally large CNV lesions that formed over large choroidal vessels. The CD31⁺ CNV lesion area was measured. Moreover, the rate of CNV lesion formation with neovessels per total formed lesions was determined. The proportion of CNV lesions with coverage of the entire CNV lesion with a SMA⁺ fibrovascular scaffold was determined as well. SMA immunolabeling was assessed in CNV lesions, which labels the activated adjacent RPE cells and the pericyte-like cells at the center of the CNV lesions. The relative rate of CNV lesions with the presence of a SMA⁺ scaffold at the center of a CNV lesion was determined in comparison with the experimental control groups.

CNV laser experiments and quantification methods adhered to previously published standards.⁸

Results

Inducible Inhibition, Specifically of Proliferating PDGFR β ⁺ Cells, in a Genetic Mouse Model Impairs the Formation of the SMA⁺PDGFR β ⁺ Scaffold and Neovessel Formation in Laser-Induced CNV Lesions without Affecting Macrophage Infiltration

We performed laser-induced CNV experiments using conditions that allow for the quantification of a CD31⁺ network of choroidal neovessels and of a SMA⁺PDGFR β ⁺ pericyte-like scaffold. We found that inducing laser spots at 120-mW power, 100-ms pulse duration, and 100- μ m spot size allowed us to observe well-formed CNV lesions at day 7 after laser injury. We measured the CD31⁺ area at sites of

laser injury, determined the rate of coverage of CNV lesions with the pericyte-like scaffold and the CNV lesion formation rate, and also assessed the presence of F4/80⁺ macrophages in these CNV lesions.

Laser injury induces the formation of a SMA⁺PDGFR β ⁺ scaffold at day 3 after laser injury, into which CD31⁺ endothelial cells grow from the underlying choroidal vessels and form neovessels at approximately day 4 in close proximity to this scaffold.⁴ This scaffold consists of elongated cells that express the prototypical pericyte markers SMA and PDGFR β , which we therefore describe as pericyte-like cells. Although in developmental angiogenesis pericytes are described to follow the occurrence of endothelial cell tubes to form functional vessels, in laser injury-induced neovessel formation we observed this pericyte-like scaffold before the identification of CD31⁺ (as well as CD144⁺) endothelial cells, which grew into this pericyte-like scaffold to form neovessels in evolving CNV lesions.⁴ Importantly, CD31⁺ neovessels do not extend beyond the area demarcated by this pericyte-like scaffold, which therefore defines CNV lesion size. A fully formed fibrovascular CNV lesion can be observed at day 5 after laser injury, which we assessed at day 7 (Figure 1).⁴

The RPE cells adjacent to the laser injury site are activated and express SMA early (starting at day 2 after laser injury) in response to laser injury, however, they do not express high levels of PDGFR β (Figure 1). Notably, NG2 is expressed by scaffold cells as well as by RPE cells (Figure 1). Thus, PDGFR β is expressed strongly by pericyte-like scaffold cells, but not to a similar extent by adjacent activated RPE cells, whereas SMA and NG2 are expressed by both pericyte-like cells and activated RPE cells adjacent to the laser injury site. Immunolabeling for SMA can therefore be used to assess both RPE cell activation (marking the border of the CNV lesions) and the formation of the pericyte-like scaffold in the center of CNV lesions.

Interestingly, immunolabeling for PDGFR β identified the cells of the pericyte-like scaffold in CNV lesions consistently, whereas immunolabeling for NG2 only identified a subset of these SMA⁺PDGFR β ⁺ cells (Figure 1). These findings suggest that PDGFR β could serve as a specific therapeutic target to ablate the pericyte-like scaffold cells in evolving CNV lesions without affecting surrounding RPE cells, whereas NG2 would not be a suitable target because it is not expressed by all scaffold cells and because it is also expressed by RPE cells.

Based on these observations we hypothesized that therapeutic targeting of proliferating PDGFR β ⁺ cells by blocking the PDGFR β signaling pathway can inhibit the formation of the SMA⁺PDGFR β ⁺ pericyte-like scaffold in evolving CNV lesions and that inhibiting the formation of this scaffold also would also prevent neovascularization.

To determine whether inducible targeted ablation, specifically of proliferating PDGFR β ⁺ cells, prevents the formation of this pericyte-like scaffold and subsequent laser-induced neovascularization and CNV lesion

Figure 1 A smooth muscle actin (SMA)⁺ platelet-derived growth factor receptor β (PDGFRβ)⁺ scaffold limits the area of neovascularization in laser-induced choroidal neovascularization (CNV) lesions. Representative laser-induced CNV lesions 7 days after laser injury are shown from a phosphate-buffered saline (PBS) (control)-treated 8-week-old male C57bl6/j mouse. Immunolabeling was performed for neovessels with the endothelial cell marker CD31 (red arrow), which labels newly formed vessels in CNV lesions. This staining was combined with antibodies against SMA (yellow arrows or blue arrows), NG2 (green arrows), or PDGFRβ (white arrows). These markers label pericyte-like cells in the center of the lesion that surround neovessels and form a central pericyte-like scaffold (SMA staining, long yellow arrows; PDGFRβ staining, long white arrow; and NG2 staining, long green arrows). This pericyte-like scaffold is particularly pronounced at the margins of CNV lesions and demarcates the area of neovascularization (SMA staining, short yellow arrows; PDGFRβ staining, short white arrow). **A:** Notably, although the pericyte-like scaffold is consistently labeled for PDGFRβ and SMA, NG2 immunolabeling is positive only for some SMA⁺PDGFRβ⁺ cells. Thus, PDGFRβ immunolabeling shows the pericyte-like scaffold in CNV lesions, whereas immunolabeling for SMA allows assessment of both scaffold formation as well as adjacent RPE activation. The findings show that PDGFRβ is a suitable target to inhibit the pericyte-like cells selectively in the center of the CNV lesion without affecting the adjacent RPE cells. PDGFRβ⁺ cells are shown in green; SMA is shown in red; and NG2 is shown in white. **B:** CD31 neovessels do not extend beyond the SMA⁺PDGFRβ⁺ pericyte-like scaffold. Notably, laser-induced injury leads to activation of RPE cells adjacent to the site of injury and CNV lesion formation, which increases SMA and NG2 expression in those RPE cells, but not PDGFRβ expression (SMA staining, blue arrows; NG2 staining, short green arrows). SMA is shown in red, NG2 is shown in white, and CD31 is shown in green. Original magnification, ×20 (confocal microscopy images).

formation, we used transgenic mice that express a viral TK under the control of the *Pdgfrb* promoter (PDGFRB-TK mice).⁵ In these mice, only PDGFRβ⁺ cells express TK, and treatment with GCV results in selective ablation of proliferating PDGFRβ⁺ cells due to incorporation of the GCV nucleoside analog during replication, leading to irreversible DNA synthesis arrest.

We treated PDGFRB-TK and control wild-type littermates with GCV daily starting on the day of laser injury (day 0 to day 6), and CNV lesions were assessed 7 days after laser injury. GCV treatment in wild-type control mice had no effect on CNV lesion formation or macrophage infiltration into evolving CNV lesions (Figure 2A). Thus, GCV treatment of PDGFRB-TK mice might inhibit proliferating PDGFRβ⁺ cells without unexpected effects of GCV on CNV lesion formation.

Importantly, we observed a consistent and potent attenuation of the SMA⁺PDGFRβ⁺ pericyte-like scaffold and

concomitant CD31⁺ neovessel formation in the center of evolving CNV lesions of PDGFRB-TK mice treated with GCV (Figure 2B). Immunolabeling for SMA in GCV-treated wild-type mice shows that CNV lesions have strongly SMA⁺-activated RPE cells adjacent to the SMA⁺ pericyte-like scaffold at the center of CNV lesions (Figure 2A). In contrast, GCV-treated PDGFRB-TK mice maintained SMA⁺-activated RPE cells (which are PDGFRβ⁻ and therefore would not be expected to be affected by GCV treatment in these mice), but lack the central SMA⁺PDGFRβ⁺ pericyte-like scaffold at the center of CNV lesions (Figure 2B) (SMA immunolabeling demarcates activated RPE cells from the central scaffold of the CNV lesion). Consistent with a critical role of the pericyte-like scaffold for neovessel formation in CNV lesions, the lack of this scaffold in GCV-treated PDGFRB-TK mice was accompanied by a lack or significant reduction of CD31⁺ neovessel formation (Figure 2B).

Figure 2 Specific ablation of proliferating platelet-derived growth factor receptor β -positive (PDGFR β^+) cells inhibits PDGFR β^+ SMA $^+$ scaffold and neovessel formation in evolving choroidal neovascularization (CNV) lesions without impairing macrophage infiltration. Temporal-specific ablation of proliferating PDGFR β^+ cells in CNV lesions of PDGFRB—thymidine kinase (TK) mice. Administration of gancyclovir (GCV) strongly inhibits pericyte-like SMA $^+$ scaffold formation in the center of the lesion (**long white arrows**) and concomitant CD31 $^+$ neovessel formation (**short white arrows**) without preventing macrophage (F4/80 $^+$ cells, **green arrows**) infiltration into CNV lesions. Activation of RPE cells in response to laser injury is not affected by targeting proliferation of PDGFR β^+ cells because adjacent RPE cells maintain strong SMA expression (**yellow arrows**). This is consistent with the lack of strong expression of PDGFR β in RPE cells. Representative confocal microscopy images of CNV lesions 7 days after laser injury from a control wild-type (WT) mouse (**A**) and a PDGFRB-TK mouse (**B**) treated with GCV are shown. **C**: Inhibiting proliferation of PDGFR β^+ cells only during the early scaffold-forming phase after CNV injury (PDGFRB-TK mice treated with GCV on days 0 to 3) inhibits scaffold formation and neovascularization, whereas a similar effect was not observed when inhibiting proliferation of PDGFR β^+ cells only during the subsequent angiogenic phase (days 4 to 7) (all CNV lesions were assessed at day 7). A CNV lesion of a control WT mouse treated with GCV on days 0 to 3 is shown in the **left panel**. SMA is shown in red and CD31 is shown in green. Original magnification, $\times 20$ (**A–C**).

We performed a detailed analysis of CNV lesion formation in these groups of mice (Figure 3C and Tables 1 and 2). We found that in GCV-treated wild-type mice CNV lesions were covered with a central SMA $^+$ pericyte-like scaffold,

whereas the majority of CNV lesions in GCV-treated PDGFRB-TK mice lacked complete coverage of CNV lesions with this scaffold (Table 2). Moreover, the absence of this SMA $^+$ pericyte-like scaffold was strongly associated

with the absence of neovessel formation or with a significantly reduced area of neovessels (Figure 3C). Whereas all laser spots in the group of GCV-treated wild-type mice resulted in CNV lesions that formed CD31⁺ neovessels, inhibition of proliferation of PDGFR β ⁺ cells in GCV-treated PDGFRB-TK mice blocked neovessel formation and only a small proportion (19.05%) of CNV lesion areas contained neovessels (Table 1). The few CNV lesions in these mice that formed neovessels showed a strongly reduced area of neovascularization (Figure 3C).

We have previously shown that targeting macrophages can potentially inhibit CNV lesion formation.^{4,9} However, GCV treatment in PDGFRB-TK mice did not prevent infiltration of macrophages (F4/80⁺ cells) into CNV lesions (Figure 2B), suggesting that the CNV inhibitory effects observed in these mice are the result of specific inhibition of proliferating PDGFR β ⁺ pericyte-like cells in evolving CNV lesions and are not due to effects on macrophages.

These findings show that targeting proliferating PDGFR β ⁺ cells potentially inhibits both pericyte-like scaffold formation, as well as neovascularization in the laser-induced model of CNV.

Because we have observed the formation of the pericyte-like PDGFR β ⁺ scaffold during the early phase of CNV lesion formation (by day 3) and before the angiogenic phase with neovessel formation (days 4 to 6), we hypothesized that inhibiting the proliferation of PDGFR β ⁺ cells only during the early scaffold-forming phase (days 0 to 3) would

Figure 3 Quantification of neovascular area, rate of choroidal neovascularization (CNV) lesion formation, and rate of pericyte-like scaffold formation in experimental mouse groups showed that therapeutic targeting of the pericyte-like scaffold through either genetic, small-molecule, aptamer, or antibody-based approaches effectively inhibits CNV. **A** and **B**: Therapeutic targeting of platelet-derived growth factor receptor β (PDGFR β) signaling in evolving CNV lesions with the neutralizing anti-PDGFR β antibody APB5 (**B**), in comparison with isotype-matched control IgG (**A**). Inactivation of PDGFR β signaling with APB5 inhibits the formation of the pericyte-like scaffold in the center of the CNV lesion (long white arrows) and concomitantly leads to reduced CD31⁺ neovessel formation (short white arrows). RPE cell activation with SMA expression (yellow arrows) and infiltration of F4/80⁺ macrophages (green arrows) are not affected by APB5 treatment. Representative confocal microscopy images of a CNV lesion from an IgG-treated control mouse (**A**) and an APB5-treated mouse (**B**) 7 days after laser injury are shown. SMA is shown in red, CD31 is shown in green, and F4/80 is shown in white. **C**: Laser-induced CNV lesions 7 days after laser injury in 8-week-old male mice. Average circumferential CD31⁺ area at sites of laser injury, reflecting neovessel formation in CNV lesions, is shown. Genetic inhibition of proliferating PDGFR β ⁺ cells leads to strong reduction of neovascularization [in gancyclovir (GCV)-treated PDGFRB—thymidine kinase (TK) mice compared with GCV-treated wild-type (WT) mice]. The extent of CNV inhibition in GCV-treated PDGFRB-TK mice was similar to that observed in aflibercept-treated mice. Inhibition of neovascularization also was observed in mice treated with fovista, tivozanib, or the combination of aflibercept and fovista. A similar inhibitory effect on neovascularization also was observed in mice treated with the neutralizing anti-PDGFR β antibody APB5. Red dotted line indicates the CD31⁺ area in aflibercept-treated mice. Means $\pm$ SEM is indicated. $N = 10$ mice/group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Original magnification, $\times 20$ (**A** and **B**). Ab, antibody; PBS, phosphate-buffered saline.

Table 1 CNV Lesion Formation Rate in Treatment Groups

Treatment	CNV lesion formation (%)	<i>P</i> value
Phosphate-buffered saline	89.29	
Fovista	73.91	0.1580
Aflibercept	80.77	0.3883
Tivozanib	90.91	0.4351
Fovista plus aflibercept	56.52	0.0068
Untreated	93.75	
IgG	84.00	0.5566
APB5	68.97	0.2347
Wild type + gancyclovir	100.00	
PDGFRB–thymidine kinase + gancyclovir	19.05	<0.0001

Percentages of formed choroidal neovascularization (CNV) lesions with neovascularization (with CD31⁺ neovessels) relative to total SMA⁺ CNV lesions are shown. *N* = 10 mice/group. *P* values compared with control groups are indicated.

be sufficient to prevent scaffold formation and CNV. In contrast, we hypothesized that inhibiting the proliferation of PDGFRβ⁺ cells only during the angiogenic phase (days 4 to 6) would be less effective in inhibiting scaffold formation and neovascularization. To test this hypothesis we treated PDGFRB-TK and WT control littermate mice daily with GCV either during the early scaffold-forming phase (days 0 to 3) or during the angiogenic phase (days 4 to 6).

We found that inhibiting proliferation of PDGFRβ⁺ cells during the early phase (days 0 to 3) was sufficient to prevent scaffold formation and neovascularization, whereas scaffold formation and neovascularization could occur when inhibiting proliferation of PDGFRβ⁺ cells only during the angiogenic phase (days 4 to 7) (Figure 2C).

Effects of Therapeutic Approaches to Block PDGFRβ-Signaling on the Formation of the Pericyte-Like Scaffold and Neovascularization

Based on our observation that genetic targeting of proliferating PDGFRβ⁺ cells can strongly inhibit laser-induced CNV lesion formation, we hypothesized that therapeutic inactivation of PDGFRβ⁺-signaling with pharmacologic inhibitors may inhibit CNV lesion formation. Our findings suggest a dependency of neovessel formation in evolving CNV lesions on this pericyte-like scaffold. Thus, we compared the inhibitory effects of different independent experimental approaches to block PDGFRβ⁺-signaling in pericyte-like cells with the effects of anti-VEGF-A therapy (which inhibits neovessel formation through inhibition of endothelial cell migration and proliferation) on CNV lesion formation. We treated mice with the anti-VEGF-A agent aflibercept, which sequesters circulating VEGF-A and is currently a standard treatment regimen for patients with neovascular AMD (aflibercept inhibits both mouse and human VEGF-A and thus can be used in our laser-induced CNV model).

We found that neutralization of VEGF-A with aflibercept significantly reduced the formation of CD31⁺ neovessels in laser-induced CNV lesions compared with control mice (Figures 3C and 4, A and B). These findings are consistent with previous experiments that used anti-VEGF-A therapies in laser-induced CNV experiments in mice, and are reflective of the beneficial effects of anti-VEGF-A therapies in patients with neovascular AMD.^{1,10} As expected, aflibercept treatment did not ablate the SMA⁺PDGFRβ⁺ scaffold (Figure 4B and Table 2), consistent with its specific effect in neutralizing VEGF-A (and therefore endothelial cell migration and proliferation) but not PDGFRβ signaling. Notably, inhibiting proliferation of PDGFRβ⁺ cells in GCV-treated PDGFRB-TK cells showed a similarly potent reduction of CNV lesion size as in those mice treated with aflibercept (there was no statistically significant difference in the CD31⁺ neovascular area between these groups of mice; *P* = 0.3342).

These findings suggest that therapeutic targeting of PDGFRβ signaling with pharmacologic agents may inhibit neovessel formation in laser-induced CNV lesions with similar efficacy as when using anti-VEGF-A therapies.

To test this hypothesis we used independent pharmacologic approaches to inhibit PDGFRβ signaling to obtain a comprehensive evaluation of the importance of the pericyte-like scaffold for CNV formation without being limited by a single treatment approach. Thus, we targeted PDGFRβ signaling during laser-induced CNV lesion formation with

Table 2 Attenuation of the Pericyte-Like SMA⁺ Scaffold in the Center of CNV Lesions of the Experimental Mouse Groups

Treatment	SMA coverage rate (% to control group)	<i>P</i> value
Phosphate-buffered saline	100.00	
Fovista	42.92	<0.0001
Aflibercept	84.38	0.1069
Tivozanib	64.48	0.0030
Fovista plus aflibercept	40.10	<0.0001
Untreated	100.00	
IgG	63.93	0.3115
APB5	36.53	0.0521
Wild type + gancyclovir	100.00	
PDGFRB–thymidine kinase + gancyclovir	16.00	<0.0001

SMA staining was used to distinguish activated SMA⁺ retinal pigment epithelium (RPE) cells adjacent to the choroidal neovascularization (CNV) area from the central pericyte-like SMA⁺ scaffold. CNV lesion sites were assessed for the presence or absence of a SMA⁺ scaffold that covered the central part of the CNV lesion. Values are indicated as a percentage compared with control groups. As expected, inhibition of vascular endothelial growth factor A signaling with aflibercept had no statistically significant effect on pericyte-like SMA⁺ scaffold formation. In contrast, inhibiting platelet-derived growth factor receptor β (PDGFRβ) signaling in either the fovista, APB5, tivozanib, or gancyclovir-treated PDGFRB–thymidine kinase experimental mouse groups strongly inhibited the formation of a pericyte-like SMA⁺ scaffold. *N* = 10 mice/group. *P* values compared with control groups are indicated.

Figure 4 Targeting vascular endothelial growth factor (VEGF)-A signaling inhibits neovascularization without affecting pericyte-like scaffold formation, whereas targeting platelet-derived growth factor receptor β (PDGFR β) signaling inhibits both scaffold formation and neovascularization. **A:** Control mice with phosphate-buffered saline (PBS) treatment only develop normal choroidal neovascularization (CNV) lesions with prominent CD31⁺ neovessels (**short white arrow**) and a SMA⁺ pericyte-like scaffold (**long white arrow**). **B:** Aflibercept (inhibiting VEGF-A signaling) reduces CNV lesion size and CD31 neovessel formation (**short white arrow**), but CNV lesions maintain coverage of CNV area with a SMA⁺ scaffold (**long white arrow**). In contrast, treatments with tivozanib [inhibiting signaling through both VEGF-A and platelet-derived growth factor receptors (PDGFRs)] (**C**), fovista (inhibiting mainly PDGFR β signaling) (**D**), or the combination of fovista and aflibercept (**E**) reduces coverage of the CNV lesions with a SMA⁺ scaffold with a concomitant reduction in CNV lesion size and neovascularization. These findings suggest that therapeutic targeting of PDGFR β signaling can reduce both scaffold formation as well as neovascularization. SMA is shown in red and CD31 is shown in green. **A–E: Long white arrows** indicate the center of CNV lesions that have a significant reduction of SMA⁺ staining in the groups treated with fovista, tivozanib, or the combination of fovista and aflibercept. **Yellow arrows** indicate activated SMA⁺ RPE cells adjacent to the laser injury site, whose activation was not affected by either treatment. Although the SMA⁺ coverage of the center of the CNV lesion is reduced in the fovista- or tivozanib-treated groups, the surrounding RPE cells remain SMA⁺. **Short white arrows** show CD31⁺ neovessels at the center of CNV lesions. Original magnification, $\times 20$ (**A–E**).

the following: i) a neutralizing antibody that is specific against PDGFR β (APB5), ii) an anti-PDGF-AB/BB aptamer (fovista), and iii) a small-molecule pharmacologic inhibitor of both VEGFRs and PDGFRs (tivozanib).

Treatment of mice with the neutralizing anti-PDGFR β antibody APB5 impaired formation of the pericyte-like scaffold in the center of CNV lesions and thereby strongly

inhibited neovessel formation (Figure 3, A and B). These findings closely resembled our observations in GCV-treated PDGFRB-TK mice, providing independent confirmation that specific inhibition of PDGFR β -signaling can inhibit scaffold formation and CNV. Quantification of CD31⁺ neovascular lesion size also showed that APB5 treatment had a similar inhibitory effect on CNV lesion size as

aflibercept treatment (there was no statistically significant difference in the CD31⁺ neovascular area between these groups of mice; $P = 0.1634$) (Figure 3C).

The pegylated DNA-based aptamer fovista (E10030) binds to PDGF-AB or PDGF-BB with high affinity and inhibits their interactions with both PDGFR α and PDGFR β .^{11,12} Thus, fovista is expected to inhibit PDGF-AB/BB-induced PDGFR β signaling in pericytes.¹²

We tested whether therapeutic inhibition of PDGFR β signaling with fovista could inhibit the formation of the PDGFR β ⁺ pericyte-like scaffold and concomitantly laser-induced CNV. We observed a significant inhibition of CD31⁺ neovessel formation in mice that were treated with fovista when compared with vehicle-treated mice (Figures 3C and 4D). CNV lesion size showed no statistically significant difference in mice treated with fovista compared with mice treated with APB5 antibodies ($P = 0.7602$).

Importantly, fovista treatment resulted in strong attenuation of SMA⁺PDGFR β ⁺ scaffold formation with loss of staining for SMA and PDGFR β in the central part of the laser injury site (whereas SMA expression in the activated RPE that does not express PDGFR β adjacent to the laser injury site was maintained), similarly as observed in PDGFRB-TK mice that were treated with GCV or in mice treated with APB5 (Figures 2B, 3B, and 4D and Supplemental Figure S1). Whereas aflibercept treatment did not affect SMA⁺PDGFR β ⁺ scaffold formation, a highly statistically significant reduction in this scaffold formation was observed for fovista (Table 2 and Supplemental Figure S1). Moreover, co-immunolabeling for NG2 and SMA showed that SMA⁺NG2⁺ cells in the center of CNV lesions were absent after fovista treatment (Supplemental Figure S1), which was expected because NG2⁺ cells of the CNV scaffold were also SMA⁺ (and thus SMA staining is a consistent marker for pericyte-like cells in the center of CNV lesions).

These findings from GCV-treated PDGFRB-TK mice and from mice treated with either APB5 or fovista collectively support the hypothesis that specific inhibition of PDGFR β signaling can attenuate neovascularization by preventing the formation of the pericyte-like scaffold in laser-induced CNV lesions.

We next assessed the effects of combined inhibition of PDGFR β signaling and VEGF-A signaling for CNV lesion formation in this model. We treated mice with a combination of aflibercept and fovista. We observed a strong inhibition of SMA⁺PDGFR β ⁺ scaffold formation as well as of CD31⁺ neovessel formation in this treatment group as well (Figures 3C and 4E, and Tables 1 and 2). Notably, the CNV lesion formation rate was reduced significantly in this combination treatment group (Table 1).

We also treated mice with a small chemical inhibitor that targets both of these pathways. Tivozanib (AV-951) is a potent small chemical inhibitor of VEGF-A receptors as well as of PDGF receptors.⁶ Thus, tivozanib treatment may mimic the effects of inhibition of VEGF-A signaling (eg, as

observed with aflibercept), as well as the effects of inhibiting PDGF-receptor signaling (eg, as observed with fovista). Thus, we tested the effects of tivozanib treatment on CD31⁺ neovessel formation, CNV lesion formation, as well as SMA⁺PDGFR β ⁺ scaffold formation. We observed that treatment with tivozanib strongly inhibited the formation of the SMA⁺PDGFR β ⁺ scaffold, similarly as observed with fovista or APB5 treatment, or in GCV-treated PDGFRB-TK mice (Table 2, Figure 4C, and Supplemental Figure S1). Consistent with a critical role of this scaffold for neovessel formation and the VEGF-A-receptor inhibitory activity of tivozanib, we also observed that this treatment potently inhibited CD31⁺ neovessel formation and overall CNV lesion size, similarly as observed in the aflibercept treatment group (there was no statistically significant difference in the CD31⁺ neovascular area between the groups of mice treated with aflibercept or tivozanib; $P = 0.7928$) (Figure 3C).

These findings suggest that combined inhibition of VEGF-A signaling (which inhibits endothelial cell migration and proliferation) and PDGFR β signaling (which inhibits pericytes and SMA⁺PDGFR β ⁺ scaffold formation) may offer a novel therapeutic strategy to potentially reduce CNV lesion size.

Notably, no major RPE abnormalities were observed in any of the treatment groups, suggesting that the treatments did not cause RPE toxicity at the administered concentrations.

Discussion

Our findings in the experiments with PDGFRB-TK mice treated with GCV that specifically target proliferating PDGFR β ⁺ cells show a critical role of PDGFR β signaling for laser-induced CNV lesion formation. Moreover, our findings suggest that targeting scaffold formation early on may prevent progression of CNV. Importantly, the genetic ablation of proliferating PDGFR β ⁺ cells in GCV-treated PDGFRB-TK mice mirrored the findings in CNV lesions of wild-type mice with pharmacologic inhibition of PDGFR β signaling, such as in mice treated with the aptamer fovista or the neutralizing anti-PDGFR β antibody APB5. In these experimental groups, CNV lesions at the site of laser injury lacked complete coverage with a SMA⁺PDGFR β ⁺ pericyte-like scaffold concomitant with reduced CD31⁺ neovessel formation in these CNV lesions. Thus, we show through independent experimental approaches that therapeutic targeting of pericyte-like cells by inhibition of PDGFR β signaling results in a potent reduction of laser-induced CNV, similarly as observed with anti-VEGF-A therapies.

Consistent with our results, it has been reported that tivozanib can inhibit laser-induced neovascularization, but our findings suggest that this effect may not only be due to inhibition of VEGF-A-receptor signaling, but may also in part be mediated through inhibition of the formation of a SMA⁺PDGFR β ⁺ scaffold.⁷

Although an important role of pericytes for pathologic neovascularization or tumor angiogenesis has been reported,^{5,13,14} the effects of anti-PDGFR β and anti-VEGF-A therapies for scaffold formation and the role of this PDGFR β ⁺ scaffold for CNV have not been assessed before. Our observations provide a novel direct link between the formation of a SMA⁺PDGFR β ⁺ pericyte-like scaffold and subsequent neovessel formation in CNV. These findings show that neovascularization in laser-induced CNV lesions requires the formation of such a scaffold and that preventing its formation can potentially inhibit pathologic neovascularization and CNV lesion formation.

In contrast to the chronic disease course observed in neovascular AMD, the laser-injury CNV model represents an acute injury model of neovascularization. To what extent our observations in the preclinical laser-injury-induced CNV model apply to neovascular AMD in patients and how PDGFR β signaling blockade with or without VEGF-A inhibition affects AMD disease progression are critical questions that need to be assessed in clinical trials. Notably, preliminary data from ongoing clinical trials have been reported to show beneficial additive effects of fovista treatment in combination with anti-VEGF-A therapies in patients with neovascular AMD.^{12,15}

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpathol.2016.02.018>.

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