Inhibition of Placenta Growth Factor Reduces Subretinal Mononuclear Phagocyte Accumulation in Choroidal Neovascularization

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Purpose. The cellular immune response driven by mononuclear phagocytes (MPs) is crucial for choroidal neovascularization (CNV) progression. Case reports show that a switch from pure anti-vascular endothelial growth factor-A (VEGF-A) intravitreal treatment to aflibercept, a drug with combined anti-VEGF-A and anti-placenta growth factor (PIGF) activity, can be beneficial for patients who do not respond to anti-VEGF-A alone. Since MPs harbor VEGFR1, we hypothesize that the interplay of P1GF/vascular endothelial growth factor receptor 1 (VEGFR1) in immune cells plays a pivotal role for CNV.

Methods. CNV was induced with laser, and immune cells and neovascularization were analyzed in vivo and ex vivo. Immunohistochemistry was employed for protein detection. Differential expression of angiogenic factors and macrophage polarization markers were assessed by quantitative PCR (qPCR). One day after laser, intravitreal injection of aflibercept or anti-PlGF was performed.

Results. In the early inflammatory phase after laser, Plgf but not Vegfa was significantly upregulated. VEGF-A upregulation is limited to the scar, whereas PlGF shows a wider distribution. M1 (proinflammatory) macrophage markers were upregulated in the early phase of CNV. However, M2 (proangiogenic) markers showed more inconsistent dynamics.

We demonstrated that both aflibercept and anti-PlGF treatments decrease the overall amount of activated subretinal MPs, and especially of those expressing PlGF. These data correlated with a reduction in leakage associated to CNV. Aflibercept showed a stronger reduction in both parameters.

Conclusions. The results hint at an interplay between P1GF/VEGFR1 and MPs that is important in the early phase of CNV. A combined inhibition of VEGF-A and PIGF is superior to a specific anti-PlGF treatment in terms of subretinal MP recruitment.

Keywords: wet AMD, choroidal neovascularization, inflammation, mononuclear phagocytes, placenta growth factor, aflibercept

Wet age-related macular degeneration (AMD) has a high economic and epidemiologic impact in the Western hemisphere, being one of the major causes for vision loss among elderly people. Current AMD treatments are based on local vascular endothelial growth factor A (VEGF-A) blockade.1 Bevacizumab, a recombinant humanized monoclonal antibody, binds all VEGF-A isoforms. Ranibizumab is a humanized antigen-binding fragment with higher affinity for VEGF-A. Aflibercept, in contrast, is a fusion protein consisting of ligand-binding elements vascular endothelial growth factor receptor (VEGFR)-1 and VEGFR2, and works as a decoy receptor binding not only VEGF-A, but also VEGF-B and placenta growth factor (PIGF).2,3 There are no remarkable differences detectable among these agents regarding their clinical efficacy in randomized clinical trial settings.1 Nevertheless, there are case reports about patients who do not respond well to certain anti-VEGF-A therapies or develop tolerance during the treatment course. In some cases, patients benefit from switching from a pure anti-VEGF-A therapy (ranibizumab, bevacizumab) to aflibercept, a therapy that combines anti-VEGF-A with anti-PlGF activity.4-14 This raises the question whether PIGF plays a specific role in the course of neovascular AMD. PIGF has been extensively characterized with regard to its role in pathologic neovascularization, in the setting of tumor growth and wound healing in particular.15,16 Literature concerning its role in retinal pathology is sparser, although it has been reported that mice lacking PIGF show less neovascularization after laser impact.17 Another group demonstrated similar results after pharmacologic blockade of PIGF.18 Whereas VEGF-A activates both VEGFR1 and VEGFR2, PIGF is a specific agonist for VEGFR1. VEGFR1 blockade led to reduction in subretinal neovascularization experimentally.19 VEGFR1 is expressed by endothelial cells,20 retinal pigment epithelium (RPE),21 and mononuclear phagocytes (MPs) (e.g., microglia, macrophages).22 VEGFR1/PIGF interaction has been described as chemoattractive in regard to macrophage recruitment,17,23 and it is important to remark that MPs play a crucial, although not fully understood, role in the development of choroidal neovascularization (CNV). Extensive study of different signaling pathways, such as CCL-2 and CCR-2, has proven...
that macrophage recruitment has an impact on CNV progression during experimental neovascular AMD. Deficiency and polymorphisms of CX3CR1, for instance, lead to altered recruitment of subretinal MPs and thus contribute to AMD progression by hampering the physiological phagocytic activity for tissue maintenance and inducing higher cytokine production.\textsuperscript{24-29} Interestingly, when VEGFR1 was blocked, the infiltration of MPs was substantially reduced.\textsuperscript{30} Thus PIGF-dependent VEGFR1 signaling might be a relevant therapeutic target in retinal inflammation.

In this study we used laser-induced CNV to examine the influence of PIGF on MP recruitment, based on the assertion that PIGF, as a ligand of VEGFR1, is crucial for neovascularization in AMD. Furthermore, we investigated whether VEGFR1/PIGF activation determines the activation of distinct subsets of MPs involved in subretinal inflammation and neovascularization. Specific inhibition of PIGF was less effective in reducing both MP recruitment and leakage associated with CNV than aflibercept, pointing toward a synergistic role of PlGF and VEGF-A via VEGFR1.

\section*{Materials and Methods}

\subsection*{Animal Model}

All animal experiments were designed and conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the governmental authorities (LAGeo, G0103/14, Berlin).

B6N.Cg-Tg (Csf1r-EGFP)1Hume/J mice are referred to as MacGreen.\textsuperscript{31} Transgenic MacGreen mice have an enhanced green fluorescent protein (EGFP) reporter under the Csf1r promoter. Thus all myeloid-derived macrophages are GFP positive. C57BL/6J mice (Charles River, Erkrath, Germany) were used as controls. All animals undergoing laser-induced CNV were 10 to 15 weeks old, weighed over 20 g, and tested negative for RD8 mutation.\textsuperscript{32}

\subsection*{Laser-Induced Choroidal Neovascularization}

After application of 2.5\% phenylephrine-hydrochloride and 0.5\% Tropicamid (Charitéapotheke, Berlin, Germany) for pupil dilatation, animals were anesthetized with intraperitoneal injection of a mix of 1\% ketamine hydrochloride (Actavis, Munich, Germany) and 0.1\% xylazine (Rompun; Bayer Vital GmbH, Leverkusen, Germany) at a dosage of 0.1 mL/10 mg body weight. A glass coverslip was lubricated with Corneregel (Bausch & Lomb GmbH, Berlin, Germany) and used as a lens before performing four or five well-distributed laser spots surrounding the optic nerve head with an argon laser (120 mW, Bausch & Lomb GmbH, Berlin, Germany) and used as a lens of 50\times magnification, Heidelberg, Germany) was settled with a c-curve of 3.5\% and incorporated a lens of 50\%.

Eyes were examined with the FA mode (diode laser, wavelength of 488 nm) at different time points (before laser and day D4, D7, and D14 after laser) distinguishing between inner and outer retina by adjusting the knob at the setup.\textsuperscript{35} Fundus angiography (FAG) was carried out 5 minutes after subcutaneous injection of fluorescein (5 mg/kg, Fluorescein 10\%; Alcon, Freiburg, Germany) and served to evaluate vascularity and leakage. Images were analyzed using Heidelberg Eye Explorer 1.7.0.0 software. Quantification of leakage in FAG images was performed in a masked fashion using ImageJ (National Institutes of Health, Bethesda, MD, USA).

\subsection*{Analysis of Retinal Whole Mounts}

Eyes were enucleated at time points before laser and D4, D7, or D14 after laser, and fixed for 13 minutes in paraformaldehyde 4\% at room temperature (RT). A peripheral incision along the limbus line permitted the removal of the cornea and the lens. The posterior eyecup was subjected to four radial cuts allowing its flattening. The neural retina was separated from the sclera by sectioning the optic nerve. Both retinal and scleral samples were permeabilized overnight with tris-buffered saline supplemented with 5\% Triton X-100. After repeated washes, samples were blocked using normal goat serum and incubated with the desirable primary antibody overnight at 4\°C (Supplementary Table S1). Species-appropriate secondary antibodies were applied for 1 hour at RT. Tissue was mounted onto glass slides and subjected to imaging with a LSM 510 confocal microscope (Zeiss, Jena, Germany). Subretinal mononuclear phagocytes associated to laser scar were counted manually in a masked fashion. CNV volume was detected using IsolectinB4 (IB4), a vascular endothelial marker, and was analyzed by sets of images (z-stack mode) and quantified using ImageJ.

\subsection*{Immunohistochemistry on Paraffin Sections}

Eyes were enucleated, fixed in Davidson fixative, and embedded in paraffin. Sections of 5-\textmu m thickness were deparaffinized and rehydrated. Different unmasking treatments were applied before blocking the samples with BSA 5\% (Supplementary Table S1). Primary antibodies (listed in Supplementary Table S1; all antibodies were validated by the supplier) were incubated overnight, followed by multiple washes and incubation with species-appropriate secondary antibodies. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (3\textmu M; Invitrogen, Carlsbad, CA, USA). Isotype controls were evaluated as shown in Supplementary Figure S2. Imaging was performed using an Axio Imager M2 fluorescence microscope (Zeiss) with ZEN lite 2012 software (Zeiss).
RNA Isolation and Quantitative RT-PCR (qPCR)

Enucleated eyes, or cell lysates for the in vitro studies, were preserved at −80°C. For RNA isolation, retinas were dissected and placed in lysis buffer. RNA extraction was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany). Quantitect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis.

For quantification, samples were run on a Rotor Gene Q (Qiagen) using QuantiNova SYBR Green RT-PCR Kit (Qiagen) for Il4r, Cd206, and Arg1 and QuantiTect SYBR Green PCR Kit (Qiagen) for all other markers tested. All reactions were prepared in triplicate. Gene expression levels were normalized to Gapdh expression. Target gene expression was analyzed using the comparative Ct (cycle threshold) method. Primer sequences are listed in Supplementary Table S2.

Statistical Analyses

Results are presented as mean ± SEM. N numbers represent the number of animals unless indicated differently, and all experiments were repeated at least three times. Experimental data were analyzed using analysis of variance (ANOVA), and P values of different treatments were evaluated using Dunnett’s test. When comparing only two groups, statistical analysis was performed using unpaired t-test. Values with statistical significance are indicated as * when P < 0.05, ** when P < 0.01, and *** when P < 0.001.

RESULTS

Placenta Growth Factor Under Control and Laser CNV Conditions

Vascular endothelial growth factor A was weakly expressed in vessels of the adult nonlasered retina (Fig. 1A), but was present in the RPE. In laser-treated eyes, marked VEGF-A expression was present at the lesion site in the subretinal space (Fig. 1B). Immunostaining of sagittal sections further confirmed VEGF-A expression in the scar (Fig. 1C).

Placenta growth factor, on the other hand, was expressed in vessels of nonlasered adult healthy retina (Fig. 1D). After laser impact, we found no difference in the expression of PlGF in vessels. PlGF expression was detectable in the subretinal space at the laser scar site at time points corresponding with macrophage recruitment and new vessel growth, analogous to VEGF-A expression (Fig. 1E). Interestingly, PlGF expression in sagittal sections was not limited to the lesion, but was also detectable in peripheral areas corresponding with vascular plexus, far from the laser scar, predominantly in the outer plexiform layer (OPL) (Fig. 1F).

Expression levels of Plgf and Vegfa together with their corresponding receptors were analyzed across the time course of CNV formation (Figs. 1G–J). No significant upregulation in Vegf expression was observed in the whole retina lysates (Fig. 1G). A significant increase in Plgf expression was observed 1 day after laser (D1) and returned to basal levels by D4 (Fig. 1H). Flt1 showed a similar pattern to sFlt1, with significant upregulation at D10 (Figs. 1I, 1J). Vegfb levels were unaltered after laser (Supplementary Fig. S5).

Mononuclear Phagocytes in CNV

IBA-1 is a marker for MPs ex vivo (Supplementary Fig. S4A). In the untreated retina, MPs presented a ramified phenotype, while during the early inflammatory phase after laser, they switched to a more amoeboid shape defined as the activated phenotype (Fig. 2A). Furthermore, a subset of MPs that was exclusively detected in the subretinal space of lasered retinas expressed PlGF. This subset of PlGF-positive amoeboid MPs was found not only at the scar site but also in peripheral regions of the subretinal space, attached to the RPE (Fig. 2B).

Expression of MP markers pointing to M1 polarization (Cd68, Cd86, Il1b, Ifnγ) was significantly upregulated in the initial inflammatory phase after laser (D1–D4; Figs. 2C–F). On the other hand, M2 markers (Il4r, Cd206) showed a decrease in expression, being significant at D14 (Figs. 2G, 2H). Arg1, another M2 marker, was significantly upregulated at D4, but decreased at later time points (Fig. 2I).

Effect of Anti-PlGF and Aflibercept on CNV

Differential gene expression was evaluated comparing the different treatments (PBS, aflibercept, or anti-PlGF antibody) to IgG control. Expression of Vegfa, Plgf, and its receptor, Flt1, was reduced significantly after anti-PlGF antibody or aflibercept injection (Figs. 3A–C). Soluble Vegfr1 (sFlt1) was unaltered among the different treatment groups (Fig. 3D).

Leakage was evaluated in vivo among the different treatment groups at D7 after laser by FAG (Figs. 3E–H). Animals that underwent aflibercept treatment had a significantly smaller leakage area compared to both controls. Eyes injected with anti-PlGF antibody showed a smaller leakage area than the control groups, but larger than the ones treated with aflibercept. However, no significant differences were found between anti-PlGF antibody and controls (IgG and PBS) or aflibercept, respectively (Fig. 3I).

CNV volume was verified in sclera flat mounts stained against IB4 (Fig. 3J). Eyes injected with either aflibercept or anti-PlGF antibody showed a significantly reduced CNV volume with respect to IgG-injected controls. When compared to PBS controls, only aflibercept-injected animals showed a significant decrease of the CNV volume.

PLGF-Positive Immune Cells

MPs in transgenic MacGreen mice were analyzed in vivo by means of their EGFP fluorescence. Immunohistochemical analysis proved that GFP is coexpressed in IBA1- and CD11b-positive cells (Supplementary Figs. S4A, S4B). Fundus autofluorescence (FA) images revealed no remarkable differences, at different time points after laser, between the anti-PlGF antibody and aflibercept groups except for the disappearance of the autofluorescence scar in eyes treated with aflibercept (Figs. 4A–D). The IgG- and PBS-injected control groups, however, had more GFP-positive cells in general throughout the time course.

Ex vivo quantitative analysis of the subretinal MPs related to the scar site at D14 after laser demonstrated significant differences between the groups in terms of cell numbers and activation status (Figs. 4E–H). Control groups showed the highest total of activated PlGF-positive myeloid cells. Anti-PlGF treatment significantly reduced these numbers. Aflibercept animals displayed the least amount of PlGF-positive immune cells (Fig. 4I). The analysis of the total amount of GFP-positive immune cells showed similar results to the double GFP- and PlGF-positive subset. Furthermore, GFP-positive ramified cells were likely not expressing PlGF (Supplementary Fig. S5).

We demonstrated in vitro that monocytes isolated from bone marrow express Vegfa, Plgf, and Flt1. After application of lipopolysaccharide (LPS) to mimic inflammation, Vegfa and Plgf expression were upregulated whereas Flt1 was significantly downregulated (Supplementary Methods, Supplementary Fig. S6).

Regarding M1–M2 polarization of the MPs, gene expression of most of the markers revealed no significant differences among the treated retina groups at D7 after laser (Figs. 5A–G).
PIGF and Inflammation in CNV

**FIGURE 1.** PIGF in the control and CNV retina. (A) Weak VEGF-A (red) expression in a retinal flat mount of a nonlased animal. Vessels are marked with IB4 (green). Scale bar: 100 μm. (B) Costaining against IB4 (green) and VEGF-A (red) in RPE flat mounts at D0, D4, and D14 after laser. Scale bar: 100 μm. (C) Staining of sagittal sections of the retina against VEGF-A (red). Nuclei were counterstained with DAPI. Scale bar: 50 μm. (D) PIGF (red) expression found in vessels (IB4, green) of a flat mount of a nonlased animal. Scale bar: 100 μm. (E) Costaining of RPE flat mounts against IB4 (green) and PIGF (red) at D0, D4, and D14 after laser. Scale bar: 100 μm. (F) Staining of sagittal sections of the retina against PIGF (red). Magnification of the scar (inset). Nuclei were counterstained with DAPI. Scale bar: 50 μm. (G-J) Fold change in expression of different genes involved in angiogenesis, normalized to Gapdh and compared to control. N = 6-12.
Regarding M1 polarization, downregulation was detected for Cd68 expression in the aflibercept group (Fig. 5A) and Il1b expression in the anti-PlGF group (Fig. 5C), whereas Tnfa expression was reduced among all groups (with significance only in the PBS group) when compared to IgG injection (Fig. 5D). Only Arg1 expression, a classical indicator for M2, was significantly reduced in both the anti-PlGF– and the aflibercept-treated groups (Fig. 5G).

**DISCUSSION**

This study aimed to improve the understanding of the role and importance of PlGF in neovascular AMD, under the hypothesis that PlGF coordinates MP recruitment. While VEGF-A expression is restricted to the CNV area, PlGF is expressed more generally throughout the retina. Analysis of inflammation associated with CNV revealed gene expression differences for markers of immune cell polarization during CNV. Furthermore, a subpopulation of MPs was PlGF positive and was present only after CNV. Intravitreal injection of anti-PlGF reduced significantly the number of PlGF-positive MPs in the subretinal space, although not as efficiently as aflibercept. The same occurred with leakage area associated with CNV. These data suggest an interplay between VEGFR1/PlGF and MPs that might be crucial for CNV progression.

VEGF-A expression in the retina has been extensively studied for RPE cells, Müller cells, and macrophages. Literature concerning PlGF is sparser. However, recent works exploring the role of PlGF in the retina emphasize its importance in neovascular AMD. Rakic and colleagues, in accordance with our findings, identified PlGF in the CNV lesion and found an upregulation of Plgf during the early phase after laser. Their histologic analysis of retinal sagittal sections showed a nonsignificant reduction in lesion size when compared to Plgf/C0 or wild type (WT) treated systemically with an inhibitor of VEGFR1. Concerning the neovascular area, another group found a dosage-dependent effect of systemic PlGF inhibition using a monoclonal antibody. Furthermore,
inhibiting the VEGFR1 signaling cascade by using Flt1-TK/− mice also reduced CNV area, a similar effect to that seen with Plgf/− mice or anti-PlGF therapy in WT mice. A third study analyzed systemic inhibition of VEGFR1 and VEGFR2 by means of leakage area and gene expression. Plgf expression was upregulated in the early phase after laser, whereas Vegfa levels remained unchanged throughout the experimental time course. These results are in accordance with our data, although they showed a more sustained increase in Plgf expression in general.
IgG $\equiv$ represents 50.

Representative images of subsets of subretinal macrophages in the scar site for IgG (in CNV). Interestingly, using several myeloid-specific VEGF- A studies have highlighted that M1 macrophages are associated more with the early phase of CNV whereas M2 are recruited (e.g., bone marrow-derived). There is a consensus that M2, the proangiogenic type of macrophages, support neovascularization in laser-induced CNV. Among other factors, it has been shown that PlGF upregulation promotes polarization toward M2. PlGF-positive subsets of immune cells detected in the subretinal space might be involved in polarization processes since they are observed only after laser. It is important to note that the dynamics of M1–M2 polarization in the early inflammatory phase of CNV have not yet been extensively described. A recent work by Yang and colleagues has partly characterized these dynamics by means of qPCR, using a more intense laser CNV model than ours. M1 macrophages were associated more with the early phase of CNV whereas M2 were, in general, predominant in the later phase. Our findings regarding M1 are very similar, although we found differences in M2 marker dynamics, most likely due to the usage of the much milder laser model (4 laser impacts versus 10 impacts). The lower levels of M2 markers in our scenario might be explained by lower levels of recruited immune cells.

Different stimuli polarize such cells, altering the gene expression of surface markers and cytokines. Differences in the expression profile are used to classify MPs into the so-called M1 or M2 polarized groups, although this classification has been controversially discussed for years since there is no clear evidence of functional implications. Several lines of evidence have highlighted that M1 macrophages are associated with higher levels of recruited immune cells.

The blocking capability of the employed anti-PlGF antibody has not been validated functionally, and this has to be taken as a limitation of the study. After treatment with anti-PlGF or aflibercept, gene expression levels of Plgf and Fli1 were reduced compared to controls, and only Vegfa for the case of
affibercept. The treatment effect was validated in terms of leakage inhibition in vivo and CNV volume ex vivo. Injection of anti-PIGF alone was less effective than aflibercept. The lack of significance in some gene expression changes may be explained by a dilution effect of the whole retina and by limiting the readout to a single time point. The exploration of MPs in vivo after treatment did not show apparent differences in activation among the groups, although in anti-PIGF- and aflibercept-treated animals, the autofluorescent scar was decreased and, in many cases, vanished at D14 whereas it persisted in controls. A quantitative analysis of MPs in vivo after treatment did not show apparent differences in the number of these cells at D14 after laser, and aflibercept was only ex vivo. Both treatments significantly decreased the number of PlGF-positive subsets associated with the scar was feasible in activation among the groups, although in anti-PIGF– and aflibercept-treated animals, the autofluorescent scar was diminished in activation.

FIGURE 5. Mononuclear phagocyte polarization after anti-PIGF treatment. (A–G) Bar charts represent fold change in expression, normalized to Gapdh and compared to IgG controls, of different genes involved in M1–M2 polarization. *PIGF stands for anti-PIGF. N = 5–12.

In summary, in the experimental setting of laser-induced CNV using our current readout, an anti-PIGF therapy is not sufficient to explain the switch responder cases. Nevertheless, this work presents relevant results indicating that certain subsets of MPs involved in CNV might be connected to PlGF signaling and thus susceptible to specific blockade.

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