Original Paper

Inhibition of Ocular Neovascularization by Co-Inhibition of VEGF-A and PLGF

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Key Words

Age-related macular degeneration (AMD) • Choroidal neovascularization (CNV) • Placental growth factor (PLGF) • Vascular endothelial growth factor A (VEGF-A) • Laser burn (LB) • Macrophages

Abstract

Background/Aims: Age-related macular degeneration (AMD) appears to be a disease with increasing incidence in Western countries and may develop into acquired blindness. Choroidal neovascularization (CNV) is the most frequent cause for AMD, and is commonly induced by regional inflammation. Past studies have highlighted vascular endothelial growth factor A (VEGF-A) as a major trigger for CNV. However, studies on the associated angiogenic factors other than VEGF-A are lacking. **Methods:** Here, we used a well-established laser burn (LB)-induced experimental CNV mouse model to study the molecular mechanisms underlying the development of CNV after ocular injury. We analyzed vessel density by lectin labeling. We isolated macrophages, endothelial cells and other cell types by flow cytometry, and analyzed levels of different angiogenic factors in these populations. We used antisera against VEGF-A (aVEGF) and/or antisera against placental growth factor (PLGF; aPLGF) to antagonize CNV. We used an antibody-driven toxin to selectively eliminate macrophages to evaluate the role of macrophages in CNV. We also examined expression of PLGF in macrophage subtypes. **Results:** The choroidal vessel density increased significantly 7 days after LB. LB increased significantly the levels of VEGF-A and PLGF in mouse eyes. Treatment with aVEGF significantly blunted increases in vessel density by LB. Treatment with aPLGF alone did not significantly reduce increases in vessel density. However, aPLGF significantly increased the inhibitory effects of aVEGF on vessel density increases. While VEGF-A was produced by endothelial cells, macrophages and other types at similar levels, PLGF seemed to be predominantly produced by macrophages. Selective macrophage depletion significantly reduced CNV. M2, but M1 macrophages produced high levels of PLGF. **Conclusions:** Together, our data suggest a previously unappreciated role of PLGF in coordination with VEGF-A to regulate CNV during ocular injury. Our study highlights macrophages and their production of PLGF as novel targets for CNV therapy.

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Introduction

Ocular neovascularization occurs in various ocular diseases, including proliferative diabetic retinopathy, age-related macular degeneration (AMD), retinopathy of prematurity and ocular hemangioma [1-4]. Both retinal and iris neovascularization can be induced by retinal hypoxia in diseases such as diabetic retinopathy and branch retinal vein occlusion and choroidal neovascularization (CNV) [1-4]. Most CNV occurs in macular lesions at the center of the retina and directly causes a severe loss of visual acuity [1-4]. Thus, all these diseases afflict persons in all stages of life from birth through late adulthood and account for most instances of legal blindness.

Inflammatory processes are thought to be critical in generating CNV [5-7]. Many experimental and clinical studies have indicated that the vascular endothelial growth factor A (VEGF-A) family, especially VEGF-A, is essential for promoting CNV [8-12]. This growth factor also influences the production of various pro-inflammatory cytokines. However, although anti-angiogenic treatments have proven therapeutic effects with antagonist against VEGF-A, the therapeutic outcome in patients is not always satisfactory [1-12].

The VEGF family is composed of six secreted proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PLGF) [13-15]. VEGF-A-mediated angiogenesis and neovascularization have been shown to be most important for embryonic and adult vessel formation and maintenance [16-20]. PLGF may play an important role in the pathological angiogenesis, in a coordinated way with other VEGF family members. However, the exact effect of PLGF on neovascularization may be case-dependent. For example, PLGF has been reported to either promote angiogenesis and tumor growth [21], or inhibit tumor angiogenesis by forming inactive VEGF/PLGF heterodimer [22]. There are three main subtypes of VEGF receptor (VEGFR), numbered 1, 2 and 3. Also, they may be membrane-bound or soluble, depending on alternative splicing. VEGF-A binds to both VEGFR1 and VEGFR2 [15]. VEGFR2 appears to mediate almost all of the known cellular responses to VEGF. The function of VEGFR1 is less well defined, although it is thought to modulate VEGFR2 signaling [15]. Interestingly, VEGFR1 is the unique binding receptor for PLGF. VEGFR3 mediates lymphangiogenesis in response to VEGF-C and VEGF-D [15]. Angiogenesis and vascularization are also inhibited by angiostatic factors, including soluble VEGFR1, endostatin, angiostatin, etc [15].

Here, we used a well-established laser burn (LB)-induced experimental CNV mouse model to study the molecular mechanisms underlying the CNV development after ocular injury [23, 24]. We found that the vessel density significantly increased 7 days after LB. LB significantly increased the levels of VEGF-A and PLGF in mouse eyes. Treatment with antisera against VEGF-A (aVEGF) significantly reduced increases in vessel density by LB. Treatment with antisera against PLGF (aPLGF) alone did not significantly reduce increases in vessel density. However, aPLGF significantly increased the inhibitory effects of aVEGF on vessel density increases. While VEGF-A was produced by endothelial cells, macrophages and other types at similar levels, PLGF seemed to be predominantly produced by macrophages. Selective macrophage depletion significantly reduced CNV.

Materials and Methods

Mouse manipulations

All mouse experiments were approved by the Institutional Animal Care and Use Committee at Capital Medical University (Animal Welfare Assurance). Twelve week-old female C57BL/6 mice were used in the current study. Ten mice were analyzed in each experimental condition.

LB-induced CNV model

CNV was induced by laser photocoagulation and evaluated as has been described previously [23, 24]. Briefly, laser photocoagulation (wavelength: 532 nm, 0.1 s, spot size: 75 μ m, power: 200 mW) was performed at four spots around the optic disc of one eye/mouse.

Choroidal vessel density quantification

One week after LB, the mice were perfused with 1 ml PBS containing 50 mg/ml Texas Red labeled Lycopodium Esculentum (Tomato) Lectin (Vector Labs, Burlingame, CA, USA), and the eyes were removed. The entire retina was carefully dissected from the eyecup and flat-mounted on an aqua-mount with the sclera facing downward and the choroid facing upward. Fluorescent images of the choroidal flat mounts were taken. The largest hyperfluorescent area corresponding to a photocoagulation spot for each eye was evaluated using NIH ImageJ software and subjected to quantitative analyses.

Intravitreal Injections

Intravitreal injections were performed under a surgical microscope. Animals were anaesthetized with the 2.5% anesthesia isoflurane. Pupils were dilated using 1% tropicamide and 2.5% phenylephrine (Sigma-Aldrich, St. Louis, MO, USA). Viscotears Liquid Gel and a microscope coverslip were used to improve the visibility of the fundus. A 33-gauge needle (Sigma-Aldrich) was inserted from the limbus with a 45° injection angle into the vitreous. The direction and location of the needle was monitored through the microscope. A volume of 2 µl antisera or reagents was injected using a repeating dispenser (PB-600-1; Hamilton Bonaduz, Reno, NV, USA). All procedures were conducted by the same surgeon.

Anti-VEGFa (aVEGF) and anti-PLGF (aPLGF) antisera were purchased from Sigma-Aldrich (USA) and were injected into mice at 1, 3 and 5 days after induction of LB at a dose of 10 µg in 2 µl Phosphate Buffered Saline (PBS, Sigma-Aldrich). Sham mice received PBS of same volume. Saporin-conjugated anti-CD11b antibody (20 µg/2 µl; Advanced Targeting Systems, San Diego, CA, USA) against the pan-macrophage surface marker CD11b was injected once at 1 day after LB to eliminate macrophages [25], while the control group received injection of same volume of IgG at the same time.

Flow cytometry

F4/80 and CD31-based cell analysis and sorting were performed by flow cytometry, after the cells were labeled with PEcy7-conjugated anti-F4/80 and APC-conjugated CD31 antibodies (Becton-Dickinson Biosciences, San Jose, CA, USA), or with anti-F4/80 and Pacific blue-conjugated anti-CD206 (Becton-Dickinson Biosciences). Flow cytometry was performed using a FACSaria (Becton-Dickinson Biosciences) flow cytometer. Data were analyzed and quantified using Flowjo software (Flowjo LLC, Ashland, OR, USA).

RT-qPCR

RNA was extracted from cells with RNeasy kit (Qiagen, Hilden, Germany) and used for cDNA synthesis. Quantitative PCR (RT-qPCR) were performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed with the RotorGene software accompanying the PCR machine, using 2-ΔΔCt method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to controls.

ELISA

The concentration of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PLGF in the cells was determined by corresponding ELISA Kit (R&D System, Los Angeles, CA, USA). ELISAs were performed according to the instructions of the manufacturer. Briefly, the collected condition media was added to a well coated with primary antibody, and then immunosorbed by biotinylated primary antibody at room temperature for 2 hours. The color development catalyzed by horseradish peroxidase was terminated with 2.5 mol/l sulfuric acid and the absorption was measured at 450 nm. The protein concentration was determined by comparing the relative absorbance of the samples with the standards.

Statistical analysis

All statistical analyses were carried out using the SPSS 19.0 statistical software package. All data were statistically analyzed using one-way ANOVA with a Bonferroni Correction. All values are depicted as mean ± standard deviation and are considered significant if $p < 0.05$.

Fig. 1. VEGF-A and PLGF levels increased in mouse eyes after LB. Seven days after laser burn (LB), the ocular vessel density was examined by lectin labeling. (A-B) Vessel density was examined, shown by representative images (A) and by quantification (B). (C-D) The levels of VEGF family members in mouse eyes were examined by RT-qPCR (C) and by ELISA (D). * $p < 0.05$. NS: non-significant. $N = 10$. Statistics: one-way ANOVA with a Bonferroni Correction. Scale bars are $50\mu\text{m}$.

Results

VEGF-A and PLGF levels increased in mouse eyes after LB

We used a well-established laser burn (LB)-induced experimental CNV mouse model to study the molecular mechanisms underlying the CNV development after ocular injury. Seven days after LB, the choroidal vessel density was examined by lectin labeling. We found that the LB-treated mice had significantly higher choroidal vessel density, compared to sham-treated mice (Fig. 1A-B). Thus, this model is proper for your further analyses.

Then we examined the levels of VEGF family members in mouse eyes by RT-qPCR (Fig. 1C) and by ELISA (Fig. 1D). While we did not detect differences in levels of VEGF-B, VEGF-C, VEGF-D and VEGF-E, significantly higher levels of VEGF-A and PLGF were detected in LB-treated mouse eyes, compared to controls (Fig. 1C-D). These data suggest that besides an established role of VEGF-A in the CNV, PLGF may be also involved in the pathological process.

Co-inhibition of PLGF significantly substantiated the anti-CNV effects of VEGF-A inhibition

Thus, we intravitreally injected antisera against VEGF-A (aVEGF) and/or antisera against PLGF (aPLGF) at 1, 3 and 5 days after LB to antagonize CNV, and analyzed vessel density at day 7 (Fig. 2A). We found that treatment with aVEGF significantly reduced increases in

Fig. 2. Co-inhibition of PLGF significantly substantiated the anti-CNV effects of VEGF-A inhibition. (A) Intravitreal injections of antisera against VEGF-A (aVEGF) and/or antisera against PLGF (aPLGF) at 1, 3 and 5 days after LB were performed to antagonize CNV. (B-C) Vessel density was examined, shown by quantification (B) and by representative images (C). * $p < 0.05$. NS: non-significant. $N = 10$. Statistics: one-way ANOVA with a Bonferroni Correction. Scale bars are $50\mu\text{m}$.

vessel density by LB (Fig. 2B-C). Treatment with aPLGF alone did not significantly reduce increases in vessel density (Fig. 2B-C). However, aPLGF significantly increased the inhibitory effects of aVEGF on vessel density increases (Fig. 2B-C). Thus, co-inhibition of PLGF seems to substantiate the anti-CNV effects of VEGF-A inhibition, which has not been appreciated in the previous studies.

PLGF was mainly produced by macrophages after LB

Since macrophages have been recently reported as a major source of PLGF in other disease models [26-30], we thus isolated macrophages (based on surface marker F4/80), endothelial cells (based on surface marker CD31) and other cell types (F4/80-CD31- cells) in mouse eyes by flow cytometry (Fig. 3A), and analyzed levels of VEGF-A and PLGF in these populations by RT-qPCR (Fig. 3B) and by ELISA (Fig. 3C). We found that while VEGF-A was produced by endothelial cells, macrophages and other types at similar levels (no significant difference, Fig. 3B-C), PLGF seemed to be predominantly produced by macrophages (Fig. 3B-C). These data suggest that unlike VEGF-A, PLGF is mainly produced by macrophages after LB.

Selective macrophage depletion significantly reduced CNV

Since PLGF is mainly produced by macrophages after LB, and since co-inhibition of PLGF substantiates the anti-CNV effects of VEGF-A inhibition, we hypothesize that selective macrophage depletion may significantly reduce CNV. To prove it, we used an antibody-driven toxin to selectively eliminate macrophages to examine its effects on CNV. CD11b is a specific marker for macrophages. A saporin-conjugated antibody against CD11b has been shown to efficiently eliminate macrophages [25]. We thus did single intravitreal injection of saporin-conjugated antibody against CD11b, or control solution, at 1 day after LB (Fig. 4A).

Fig. 3. PLGF was mainly produced by macrophages after LB. (A) Macrophages (based on F4/80+), endothelial cells (based on CD31+) and other cell types (F4/80-CD31- cells) in the eyes were separated by flow cytometry, shown by representative flow chart. (B-C) The levels of VEGF-A and PLGF in these populations were analyzed by RT-qPCR (B) and by ELISA (C). * $p < 0.05$. NS: non-significant. N=10. Statistics: one-way ANOVA with a Bonferoni Correction.

Fig. 4. Selective macrophage depletion significantly reduced CNV. (A) Single intravitreal injection of saporin-conjugated antibody against CD11b (saporin-anti-CD11b) to selectively eliminate macrophages at 1 day after LB. (B-C) The elimination of macrophages was confirmed by examination of macrophage percentage by flow cytometry, shown by representative flow charts (B) and by quantification (C). (D-E) Vessel density was examined, shown by representative images (D) and by quantification (E). * $p < 0.05$. NS: non-significant. N=10. Statistics: one-way ANOVA with a Bonferoni Correction. Scale bars are 50 μ m.

The elimination of macrophages was confirmed by examination of macrophage percentage by flow cytometry (Fig. 4B), showing a significant decrease (85% decrease, Fig. 4C). Further, macrophage depletion significantly reduced the increases in vessel density by LB (Fig. 4D-E).

Fig. 5. PLGF was mainly produced by M2 macrophages. (A) In order to figure out the production of PLGF by macrophage subtypes, we used a M2-specific cell surface marker CD206 to label cells in different conditions, and found that neither anti-VEGF-A treatment, nor macrophage ablation affected the ratio of M1 to M2 macrophages. However, anti-PLGF treatment significantly increased the M1:M2 ratio, suggesting that M2 macrophage polarization may be inhibited by PLGF antagonizing. (B) Significant higher levels of PLGF transcripts were detected in M2 macrophages, compared to M1. * $p < 0.05$. NS: non-significant. $N = 10$. Statistics: one-way ANOVA with a Bonferroni Correction. Scale bars are $50\mu\text{m}$.

Fig. 6. Schematic of the model. Macrophages produce PLGF and VEGF-A, and endothelial cells and other cells produce VEGF-A; VEGF-A triggers CNV, which could be substantiated by PLGF.

PLGF was mainly produced by M2 macrophages

In order to figure out the production of PLGF by macrophage subtypes, we used a M2-specific cell surface marker CD206 to label cells in different conditions, and found that neither anti-VEGF-A treatment, nor macrophage ablation affected the ratio of M1 to M2 macrophages (Fig. 5A). However, anti-PLGF treatment significantly increased the M1:M2 ratio, suggesting that M2 macrophage polarization may be inhibited by PLGF antagonizing. Moreover, significant higher levels of PLGF transcripts were detected in M2 macrophages, compared to M1 (Fig. 5B). Together, these data suggest that PLGF is mainly produced by M2 macrophages. Our study demonstrates a model in that after injury, macrophages produce PLGF and VEGF-A, and endothelial cells and other cells produce VEGF-A; VEGF-A triggers CNV, which could be substantiated by PLGF (Fig. 6).

Discussion

Ocular neovascularization can be induced by retinal hypoxia in diseases such as diabetic retinopathy and branch retinal vein occlusion and CNV [1-4]. Most CNV occurs in macular lesions at the center of the retina and directly causes a severe loss of visual acuity

[1-4]. Inflammation-induced VEGF-A production is essential for development of CNV [5-12]. However, although anti-VEGF-A treatments have proven therapeutic effects, the outcome remains unsatisfactory.

The VEGF family is composed of six secreted proteins, among which PLGF may play an important role in the pathological angiogenesis [20, 31], in a coordinated way with other VEGF family members. However, the exact effect of PLGF on neovascularization may be case-dependent.

Laser burn has been recognized as a potential trigger for CNV [32]. Here, we used a well-established laser burn (LB)-induced experimental CNV mouse model to study the molecular mechanisms underlying the CNV development after ocular injury [23, 24]. We validated the model by examination of vessel density at 7 days after LB. We used lectin labeling, rather than an endothelial cell marker, e.g. CD31, CD106 and CD144 [13-15, 33-35], since lectin likely labels functional vessels and appears to be a better choice for analyzing the functionality of the newly formed vessels [36].

Interestingly, we found that LB significantly increased the levels of VEGF-A and PLGF, but not other VEGF family members in mouse eyes. Moreover, treatment with aVEGF but not aPLGF significantly reduced increases in vessel density by LB. However, aPLGF significantly increased the inhibitory effects of aVEGF on vessel density increases. Since PLGF and VEGF-A share a same receptor, VEGFR1 [13-15], it may be expected that PLGF substantiates the effects of VEGF-A either by modulating VEGFR1-mediated downstream signaling, or by forming heterodimer with VEGF-A to modify the ligands [21, 22]. These mechanisms may be conducted through ligand (with different homodimers or heterodimer) competition for receptor (VEGFR1) binding [21, 22].

Next, we found that VEGF-A was produced by endothelial cells, macrophages and other types at similar levels, PLGF seemed to be predominantly produced by macrophages. These data suggest that macrophages may be the unique source for PLGF in the eyes after injury, while VEGF-A are from multiple sources. Specially, the VEGF-A in CD31-F4/80- cells may be produced by mesenchymal cells [13-15]. In order to confirm the role of PLGF, we selectively eliminated macrophages by a saporin-conjugated antibody against CD11b [25]. Saporin is a ribosome-inactivating protein from seeds of the plant *Saponaria officinalis* [25]. Saporin is safe for laboratory use under normal safety conditions and the LD50 in mice is 4 mg/kg. Moreover, Saporin does not have a method of cell entry on its own [25]. Thus, CD11b antibody will direct entrance of Saporin into macrophages to selectively kill them [25]. We found that macrophage depletion significantly reduced the increases in vessel density by LB, which confirmed our hypothesis. However, besides monocytes, CD11b is also expressed by dendritic cells and can be upregulated on activated cells irrespective of their naive expression status. Thus, CD11b-driven Saporin may also eliminate some dendritic cells. Future experiments may be designed to examine the effects of dendritic cell ablation on the interpretation of the results.

According to literature [26-30], the PLGF may be predominantly secreted by a subpopulation of macrophages called M2 macrophages, which are distinct from the classical M1 macrophages. We confirmed it in the current study.

To summarize, our study shows a model in that after injury, macrophages produce PLGF and VEGF-A, and endothelial cells and other cells produce VEGF-A; VEGF-A triggers CNV, which could be substantiated by PLGF.

Disclosure Statement

The authors have declared that no competing interests exist.

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