Original Paper

Combined Anti-PLGF and Anti-Endostatin Treatments Inhibit Ocular Hemangiomas

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Key Words

Placental growth factor • Endostatin • Ocular hemangiomas • Ocular neovascularization

Abstract

Background/Aims: The degree of neovascularization determines the aggressiveness of ocular hemangiomas (OH). So far, the anti-angiogenic treatments using either antagonists against vascular endothelial growth factor A (VEGF-A), or endostatin, do not always lead to satisfactory therapeutic outcome. **Methods:** We examined the VEGF receptor 1 (VEGFR1) levels in the OH specimen. We compared the effects of anti-PLGF, endostatin, as well as their combined treatments on the growth of OH in a mouse model, using bioluminescence imaging in living animals. We also examined vascularization by CD31 expression. **Results:** We detected higher VEGFR1 levels in the OH, compared to paired normal tissue. Thus, we hypothesize that as a major ligand for VEGFR1, placental growth factor (PLGF) may also play a role in the neovascularization and tumorigenesis of OH. In an implanted OH model in mice, we found that both anti-PLGF and endostatin significantly decreased OH growth as well as vascularization, while combined treatments had a significantly more pronounced effect. **Conclusion:** Our data suggest that combined anti-PLGF and endostatin may be a more effective therapy for inhibition of ocular vascularization and the tumor growth in OH.

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Introduction

Hemangioma is a non-cancerous tumor caused by abnormal growth of blood vessels. Hemangiomas can occur anywhere on the body, but are most commonly found on the face and neck [1-3]. Ocular hemangiomas (OH) can occur on the eyelids, on the conjunctiva or

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in the orbit, and may interfere with the normal development of the eye and possibly lead to vision problems, including amblyopia and glaucoma [1-3]. Ocular neovascularization may also occur in several ocular diseases, including proliferative diabetic retinopathy, neovascular age-related macular degeneration, and retinopathy of prematurity [4-7]. Together, all these diseases afflict persons in all stages of life from birth through late adulthood and account for most instances of legal blindness. Although anti-angiogenic treatments have proven therapeutic effects either with antagonist against vascular endothelial growth factor A (VEGF-A), or with endostatin, the therapeutic outcome in patients is not always satisfactory [4-12].

Angiogenesis and vascularization are promoted by angiogenic factors, including fibroblast growth factor, angiopoietins, matrix metalloproteinase (MMP), and VEGF family members, among which VEGF family is the most potent and important trigger for angiogenesis and vascularization [13]. The VEGF family is composed of six secreted proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PLGF) [13-18], which play critical roles in cancer growth and metastasis [19-25]. 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 cancer neovascularization may be case-dependent. For example, PLGF produced by tumor and host cells has been reported to promote angiogenesis and tumor growth [26], or inhibit tumor angiogenesis by forming inactive VEGF/PLGF heterodimer [27]. 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 [13]. 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 [13]. Interestingly, VEGFR1 is the unique binding receptor for PLGF. VEGFR3 mediates lymphangiogenesis in response to VEGF-C and VEGF-D [13]. Angiogenesis and vascularization are also inhibited by angiostatic factors, including soluble VEGFR1, endostatin, angiostatin, etc [13].

In the current study, we detected higher VEGFR1 levels in the OH, compared to paired normal tissue, leading to the hypothesis that as another major ligand for VEGFR1, PLGF may also play a role in the neovascularization and tumorigenesis of OH. Thus we compared the effects of anti-PLGF, endostatin, and combined treatment on the growth of OH *in vivo* in a mouse model. We found that both anti-PLGF and endostatin significantly decreased OH growth, while combined treatment had a more pronounced effect. Moreover, the inhibition of OH growth was in parallel with the inhibition on vascularization.

Materials and Methods

Cell lines and reagents

C166 is a mouse endothelial cell line purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and was established from cells from F1 embryos obtained by mating a female NMRI/GSF mouse with a male CD-1 mouse that was transgenic for the human *fes* (*fps/fes*) proto-oncogene [28]. C166 cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 15% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Anti-PLGF antisera and endostatin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Transfection of C166 cells

C166 cells were transfected with a plasmid carrying luciferase and GFP, connected by a 2A, under a CMV promoter. The small 2A peptide sequences, when cloned between genes, allow for efficient, stoichiometric production of discrete protein products within a single vector through a novel "cleavage" event within the 2A peptide sequence. Transfection was performed with Lipofectamine2000 (Invitrogen) transfection kit, according to the manufacturer's instructions. Successfully transduced C166 cells were selected by flow cytometry based on GFP.

Mouse handling and in vivo bioluminescence imaging

All mouse experiments were approved by the Institutional Animal Care and Use Committee at Shandong University (Animal Welfare Assurance). Surgeries were performed under ketamine/xylazine anesthesia, according to the Principles of Laboratory Care. Twelve week-old male NOD/SCID mice were used in the current study. Five mice were analyzed in each experimental condition. Luciferase-expressing C166 cells (10^5) were orthotopically implanted into the orbit of 12 week-old male NOD/SCID mice. After 4 weeks, the tumor growth was monitored and quantified by luminescence levels. Bioluminescence was measured with the IVIS imaging system (Xenogen Corp., Alameda, CA, USA). All of the images were taken 10 minutes after intraperitoneal injection of luciferin (Sigma-Aldrich) of 150mg/kg body weight, as a 60-second acquisition and 10 of binning. During image acquisition, mice were sedated continuously via inhalation of 3% isoflurane. Image analysis and bioluminescent quantification was performed using Living Image software (Xenogen Corp).

Western blot

The protein was extracted from the resected OH from the patient specimen, or the resected OH from the mice, using RIPA buffer (Sigma-Aldrich). The supernatants were collected after centrifugation at 12000×g at 4°C for 20min. Protein concentration was determined using BCA protein assay, and whole lysates were mixed with 4×SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100 °C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system (Pierce, Rockford, IL, USA) to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies were anti-VEGFR1 (Santa Cruz Biotechnology, Dallas, Texas, USA), anti-CD31 (Becton-Dickinson Biosciences, San Jose, CA, USA) and anti- β -actin (Cell Signaling, San Jose, CA, USA). β -actin was used as a protein loading control.

RT-qPCR

RNA was extracted from resected OH 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). Primers for CD31 and β -actin were purchased from Qiagen. Values of genes were normalized against β -actin and then compared to the control.

Statistical analysis

All statistical analyses were carried out using the SPSS 17.0 statistical software package. All data were statistically analyzed using one-way ANOVA with a Bonferoni Correction, followed by a Fisher's Exact Test for comparison between two groups. All values are depicted as mean $\pm$ standard deviation from 5 individuals and are considered significant if $p < 0.05$.

Results*High VEGFR1 was detected in OH*

Since the degree of neovascularization determines the malignancy of OH, and since present anti-angiogenesis treatments did not always achieve satisfactory results, we were prompted to figure out whether targeting angiogenic or anigostatic factors other than

Fig. 1. High level of VEGFR1 was detected in OH. Representative Western blots for VEGFR1 in the resected OH samples and paired normal tissue (NT) from the same patient. β -actin was used as a protein loading control.

Fig. 2. Preparation of GFP and luciferase co-expressing C166 cells. (A) We transduced a mouse endothelial cell line, C166, with GFP and luciferase. (B) Successfully transduced cells (green rectangle) were isolated by flow cytometry, shown by a representative flow chart. (C) Purified green fluorescent cells in culture. All green cells also co-expressed luciferase, to allow detection *in vivo* after luciferin injection. Scale bar is 50 μ m.

Fig. 3. Combined treatments with anti-PLGF and endostatin inhibited OH growth *in vivo*. We orthotopically implanted 10^5 luciferase-expressing C166 cells into the orbit of 12 week-old male NOD/SCID mice. The mice received intraperitoneal injection with either control saline, or anti-PLGF alone, or endostatin alone, or combined anti-PLGF and endostatin, every other day till 4 weeks when the mice were monitored for tumor growth. (A-B) Bioluminescence was shown by representative images (A), and by quantification (B). * $p < 0.05$. NS: non-significant. N=5.

VEGF-A and endostatin may improve the outcome of the anti-angiogenesis treatments. We analyzed the resected OH samples from the patients by Western blot, and compared to paired normal tissue (NT). We detected higher VEGFR1 levels in OH specimen (Fig. 1). VEGFR1 has two ligands, VEGF-A and PLGF. Specifically, VEGFR1 is the unique receptor for PLGF. We thus hypothesized that PLGF may also play a role in the neovascularization and tumorigenesis of OH.

Fig. 4. Combined treatment with anti-PLGF and endostatin inhibited ocular neovascularization. (A-B) The vessel density in OH from the mice was quantified by CD31 transcript levels (A) and by CD31 protein, shown by representative Western blots (B). * $p < 0.05$. NS: non-significant. N=5.

Preparation of C166 cells labeled with GFP and luciferase

Thus we aimed to compare the effects of anti-PLGF, endostatin and combined treatment on the growth of OH *in vivo* in a mouse model. First, we transduced a mouse endothelial cell line C166 with GFP and luciferase for purification and *in vivo* tracing, respectively (Fig. 2A). Successfully transduced cells were selected by flow cytometry (Fig. 2B), showing high green fluorescence in culture. These green cells also expressed luciferase, to allow detection *in vivo* after luciferin injection.

Combined treatment with anti-PLGF and endostatin inhibited OH growth in vivo

Then we orthotopically implanted 10^5 luciferase-expressing C166 cells into the orbit of 12 week-old male NOD/SCID mice. The mice received intraperitoneal injection with either control saline, or anti-PLGF alone, or endostatin alone, or combined anti-PLGF and endostatin, every other day till 4 weeks when the mice were monitored for tumor growth.

We found that both anti-PLGF and endostatin alone significantly decreased OH growth, while combined treatment had a significantly more pronounced effect (Fig. 3A-B). These data suggest that endostatin and anti-PLGF have independent anti-tumor effects and the combination has an enhanced effect than each.

Combined treatment with anti-PLGF and endostatin inhibited ocular neovascularization

Moreover, when we analyzed the vessel density in OH from these mice by quantification of CD31 at both transcription level (Fig. 4A) and protein level (Fig. 4B), we found that the inhibition of OH growth was in parallel with the inhibition on vascularization. CD31 is a specific marker for endothelial cells. These data suggest that combined anti-PLGF and endostatin may be more effective therapy for inhibition of ocular vascularization. This anti-angiogenesis effect in OH results in inhibition of the tumor growth.

Discussion

Ocular neovascularization occurs in ocular diseases, including proliferative diabetic retinopathy, neovascular age-related macular degeneration, retinopathy of prematurity, and specifically, OH [1, 2, 4-7]. Although anti-angiogenesis treatments have proven therapeutic effects with either antagonist against VEGF-A, or with endostatin, the therapeutic outcome in the patients is not always satisfactory [4-7].

VEGF family is the most potent and important trigger of angiogenesis and vascularization [13]. The VEGF family is composed of six secreted proteins, including VEGF-A and PLGF [13-15]. PLGF plays an important role in the pathological angiogenesis. Among the three VEGFRs, VEGFR1 binds to both VEGF-A and PLGF and is the only receptor that PLGF binds. In the current study, we detected high level of VEGFR1 in the OH from the patients, leading to the hypothesis that PLGF may also play a role in the neovascularization and tumorigenesis of OH.

Thus we compared the effects of anti-PLGF, endostatin, and combined treatment on the growth of OH *in vivo* in a mouse model. We found that both anti-PLGF and endostatin significantly decreased OH growth, while combined treatment had a more pronounced effect. These data suggest that both PLGF and endostatin regulate the angiogenesis of OH, but they may have different regulation pathways. We further found that the inhibition of OH growth was in parallel with the inhibition in vascularization, suggesting that inhibition of OH growth may result from the inhibition of the growth of endothelial cells and angiogenesis. Taken together, our data suggest that combined anti-PLGF and endostatin may be more effective therapy for inhibition of ocular vascularization, and specifically in OH, the tumor growth. Our result thus sheds insight on a new strategy to control the ocular neovascularization and treatment of related diseases through targeting both PLGF and endostatin.

Disclosure Statement

The authors have declared that no competing interests exist.

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