Knockdown of the Placental Growth Factor Gene Inhibits Laser Induced Choroidal Neovascularization in a Murine Model

Ramin Nourinia1, MD; Zahra-Soheila Soheili2, PhD; Hamid Ahmadian1, MD; Hassan Akrami3, PhD; Mozghan Rezaei Kanavi1, MD; Shahram Samiei4, PhD

1Ophthalmic Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2National Institute of Genetic Engineering and Biotechnology, Tehran, Iran
3Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran
4Iranian Blood Transfusion Organization, Tehran, Iran

Purpose: To evaluate the effect of placental growth factor (PIGF) gene knockdown in a murine model of laser-induced choroidal neovascularization.

Methods: Choroidal neovascularization was induced in the left eyes of 11 mice by infrared laser. Small interfering RNA (siRNA, 20 picomoles/10 μl) corresponding to PIGF mRNA was administered intravitreally by Hamilton syringe in all subjects. One month later, fluorescein angiography and histologic examination were performed.

Results: No leakage was apparent in the 11 eyes treated with siRNA cognate to PIGF. The results of histological evaluation were consistent with angiographic findings showing absence of choroidal neovascularization.

Conclusion: Knockdown of the PIGF gene can inhibit the growth of laser-induced choroidal neovascularization in mice.

Keywords: Choroidal Neovascularization; Placental Growth Factor; Small Interfering RNA

INTRODUCTION

The vascular endothelial growth factor (VEGF) is a well-recognized inciting angiogenic stimulus for the growth of choroidal neovascularization (CNV).1 The VEGF family includes VEGF-A, -B, -C, and -D in addition to the placental growth factor (PIGF). The presence of these proteins has recently been reported within human CNV membranes.2 PIGF is a glycoprotein synthesized in the inner portion of the neural retina and plays a key role in promoting monocyte chemotaxis, collateral vessel growth, and endothelial cell growth and migration.3,4 PIGF and VEGF demonstrate synergistic effects in pathological angiogenesis amplifying VEGF-driven angiogenesis and activation of vascular endothelial cells.5 Furthermore, PIGF mRNA expression is present in the intact choroid and is significantly up-regulated during experimental induction of CNV.6

RNA interference (RNAi) results in destruction of mRNAs that share homologous sequences with the double strand RNA (dsRNA). It has been established that synthetic RNAs, 21 and 22 nucleotides in length, called small interfering RNAs (siRNAs), are able to mediate cleavage of the target RNA.7 An in vitro study revealed that anti PIGF siRNA can hinder new vessel formation by inhibiting PIGF production.8
In the present study we evaluated the efficacy of siRNA directed against the PlGF gene in a mouse model of laser-induced CNV.

**METHODS**

The study was performed on 11 adult mice. All animal experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were anesthetized by a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). Pupillary dilatation was achieved by administering tropicamide 1% eye drops (SinaDarou, Tehran, Iran) prior to fundus examination, fluorescein angiography and retinal laser photocoagulation.

Choroidal neovascular lesions were induced at 1, 3, 5 o’clock positions at a distance of two disc diameters from the optic disc margin using an infrared diode laser (Keeler Microlase, Windsor, UK). Three laser burns were applied at 0.1 second duration with spot size of 100 μm and power settings ranging from 450 mW to 850 mW in order to induce a break in Bruch’s membrane indicated by the formation of a vapor bubble and a small hemorrhage. Failure to produce breaks in Bruch’s membrane or severe bleeding during laser photocoagulation resulted in exclusion of the animal from the study.

Immediately after laser photocoagulation, 11 mice received 20 picomoles /10 microliter of PlGF-specific siRNA (Qiagen, Hilden, Germany). Each siRNA solution was injected into the mid-vitreous cavity using a 10 microliter Hamilton syringe and a 30-gauge needle. Injections were monitored for evidence of reflux or inadequate injection; eyes with extensive substance reflux were excluded from the analysis.

Fluorescein angiography was performed 30 days following laser induction for each animal using the Heidelberg Retina Angiograph (HRA2, Heidelberg Engineering, Heidelberg, Germany). The angiograms were taken by intravenous injection of 10% fluorescein sodium (0.1 mL/kg).

On the same day, the animals were sacrificed under deep anesthesia and their eyes were carefully enucleated and placed in 10% neutral-buffered formalin overnight. Subsequently, each globe was processed and embedded in paraffin. Serial sections were performed and stained with hematoxylin and eosin. Slides were observed and photographed under bright field microscopy.

**RESULTS**

No choroidal neovascularization was visualized on fluorescein angiography in any of the 11 eyes that had received siRNA cognate to PlGF (Fig. 1).

![Image](image1.png)  ![Image](image2.png)

**Figure 1.** Fluorescein angiogram taken 30 days after laser injury; no leakage is observed.
Histological evaluation of the tissue samples was consistent with angiographic findings. Histologic features of CNV such as telangiectatic vessels in the choriocapillaris, subretinal hemorrhage and presence of focal subretinal exudates adjacent to the disrupted retinal pigment epithelium were not observed in any of the 11 treated eyes that had received siRNA cognate to PlGF (Figures 2A and 2B).

**DISCUSSION**

The development of choroidal neovascularization in eyes treated with intravitreal siRNA corresponding to PlGF mRNA was not observed in the current study, which indicates that siRNA cognate to PlGF may hinder formation of laser induced CNV.

Placental growth factor is thought to play an important role in the angiogenesis process; it could individually enhance angiogenesis through activation of VEGF receptor type 1 (VEGFR-1) by forming PlGF/VEGF-A heterodimers. Rakic et al demonstrated that both deficient PlGF expression and PlGF receptor neutralization can significantly reduce the incidence and severity of laser-induced CNV. PlGF and VEGF were revealed to acquire different roles during retinal vascular development that could be justified by activation of distinct receptors; VEGF-A binds to both VEGF-R1 and VEGF-R2 whereas PlGF is unable to activate VEGF-R2 which is necessary for mitogenic and proliferative responses in endothelial cells. Brave et al clarified that PlGF may play a fundamental role in driving VEGF-A dependent angiogenesis when the local concentration of VEGF is low. Choroidal PlGF levels were upregulated three days after laser injury in a study by Van de Veire and colleagues. They showed that intravitreal anti-PlGF antibody could inhibit laser induced CNV and choroidal vessel leakage. Besides, they argued in favor of the efficacy and specificity of anti-PlGF monoclonal antibody focusing on the results of their study, and emphasized that delivery of monoclonal antibody and PlGF loss could induce similar mechanisms with contextual differences. They stated that angiogenesis was inhibited comparably by loss or inhibition of PlGF in choroidal neovascularization.

VEGFR-2 expression has been detected in non-endothelial retinal cells such as ganglion cells, neural photoreceptors and Muller cells which illustrates the non-vascular function of VEGF within the retina; therefore, VEGFR-2 blockade may produce potentially harmful effects. Currently available anti-VEGF drugs for treatment of CNV do not differentiate various signaling systems mediated by VEGFR-1 and VEGFR-2. In addition, long-term exposure to VEGFR-2 blockade could have serious consequences for the retinal nerve system. These considerations along with PlGF binding specificity for VEGFR-1, makes the latter receptor a safer target for treating neovascular disorders.

It has been demonstrated that PlGF knockdown within the retinal pigment epithelium
(RPE) has no impact on the proliferation and apoptosis of these cells. This could signify minimal side effects from PlGF knockdown on the morphology and functionality of RPE cells. Consequently, suppression of PlGF gene did not affect RPE cell proliferation and survival, nor alter the transcript levels of RPE65, cellular retinaldehyde-binding protein (CRALBP) or tyrosinase in cultures treated by siRNA cognate to PlGF.8

A major restriction of intravitreal injection is the limited volume of drugs which can be administered in the confined vitreous cavity. By applying siRNA, 100 to 1000 molecules of the target protein can be inhibited by each mRNA molecule whereas only one molecule of the target protein can be inhibited by each molecule of the antibody. Efficacy of siRNA targeting VEGF in decreasing the extent of experimental CNV has already been revealed.12 In an in vitro study by Akrami et al, almost 92% of RPE cells were successfully transfected using 20 pmol/ml siRNA; analysis of PlGF gene expression by real-time reverse-transcription polymerase chain reaction (PCR) indicated that PlGF transcripts reached their lowest level (10% of controls) after 36 hours and that vascular tube formation was efficiently reduced with siRNA cognate to PlGF.8

Immune stimulation, especially involvement of toll-like receptor3 (TLR3), has recently been a concern in the RNAi field. According to a recent study, siRNA-based treatment for wet type AMD does not provoke an immune response. This study delineated that naked siRNA could enter the target cells in the eye and inhibit its target gene but was unable to activate TLR3; intravitreal injection of the siRNA, targeting RTP801 (either naked or lipofectamine formulated), resulted in detection of siRNA molecules in retinal ganglion cells, RPE and retinal and choroidal endothelial cells. However, no TLR3 receptor activation was observed (Feinstein et al, Proceedings of the Association for Research in Vision & Ophthalmology 2009, Fort Lauderdale).

In summary, we used siRNA cognate to PlGF to investigate the effect of PlGF knockdown in a murine model of laser-induced choroidal neovascularization. This was performed based on our previous in vitro study, in which we evaluated the specificity of desired PlGF siRNA and outlined no off-target effects on RPE cultures. According to our experimental study, the naked siRNA cognate to PlGF may have a significant value in the treatment of CNV due to different etiologies. However, further studies with larger sample size and a control group are required to investigate this issue.

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Conflicts of Interest
None.

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