Lack of Thrombospondin 1 and Exacerbation of Choroidal Neovascularization

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Objectives: To assess the impact of thrombospondin 1 (TSP1) deficiency on choroidal neovascularization (CNV) and to determine whether administration of a TSP1 antiangiogenic mimetic peptide attenuates CNV.

Methods: The impact of TSP1 deficiency on laser-induced CNV was assessed using wild-type (TSP1+/−) and TSP1-deficient (TSP1−/−) mice. Three laser burns were placed in each eye of TSP1+/− and TSP1−/− mice to induce CNV. Intravitreal injection of the TSP1 mimetic peptide was performed on days 1 and 7 postlaser in the mice. For quantitative measurements of neovascularization, intercellular adhesion molecule 2 staining was performed at 14 days postlaser of the choroidal-sclera flat mounts. The recruitment of macrophages to the sites of damage was investigated by immunohistochemistry. The CNV area was measured by intercellular adhesion molecule 2 staining and use of ImageJ software.

Results: The TSP1−/− mice exhibited significantly larger areas of neovascularization on choroidal flat mounts compared with TSP1+/− mice. This was consistent with enhanced recruitment of macrophages in TSP1−/− mice compared with TSP1+/− mice 3 days postlaser. The development of CNV was significantly attenuated in mice receiving the TSP1 antiangiogenic mimetic peptide compared with those receiving vehicle alone.

Conclusions: Deficiency of TSP1 contributes to enhanced choroidal neovascularization. This is consistent with the anti-inflammatory and antiangiogenic activity of TSP1. The TSP1 antiangiogenic peptide was effective in attenuation of CNV.

Clinical Relevance: Intravitreal injection of TSP1 antiangiogenic mimetic peptides may provide alternative treatment for CNV.


AGE-RELATED MACULAR DEGENERATION (AMD) affects millions of individuals worldwide, with approximately 90% of severe vision loss attributed to choroidal neovascularization (CNV).1,2 The global prevalence of CNV is expected to double in the next decade because of the aging population. Age-related macular degeneration is characterized by a progressive degeneration of the macula, usually bilateral, leading to a central scotoma and severe decrease in vision. The visual deficit results initially from retinal degeneration called geographic atrophy (dry or nonexudative AMD) often complicated by the secondary effects of CNV (wet or exudative AMD). An early sign of AMD is the appearance of drusen, which are extracellular deposits that accumulate below the retinal pigment epithelium and are known to be a risk factor for developing CNV.3

Angiogenesis, the formation of new capillaries from preexisting capillaries, is associated with the pathogenesis of exudative AMD.3,4 Therefore, inhibition of angiogenesis has become a viable strategy to inhibit CNV. Targeting of the proangiogenic factor vascular endothelial growth factor A has been validated in patients with CNV.5,6 However, significant improvement in vision is only observed in 30% of patients treated with a vascular endothelial growth factor A antagonist, with 20% of treated patients still progressing to legal blindness. Furthermore, safety concerns about the conditional blockade of vascular endothelial growth factor A, which is constitutively expressed in the normal human retina and glomeruli,9,10 are emerging. Thus, additional treatment strategies on the basis of more specific targeting of CNV are desirable.

It is now well accepted that endogenous inhibitors of angiogenesis play a sig-
significant role in regulation of angiogenesis and their decreased production may contribute to pathological neovascularization. Thrombospondin 1 (TSP1), a member of the TSP gene family, was the first endogenous inhibitor of angiogenesis identified whose expression was decreased during malignant transformation.\textsuperscript{1,2} We have shown that TSP1 is an important modulator of retinal vascular homeostasis and its increased production results in attenuation of retinal vascular development and neovascularization.\textsuperscript{3,4} We have also shown that TSP1 is present at very high levels in vitreous samples from various species and its level decreased during diabetes mellitus, perhaps contributing to the pathogenesis of diabetic retinopathy.\textsuperscript{5,6} However, TSP1 expression changes and its contribution to the pathogenesis of AMD need further investigation.

Thrombospondin 1 is synthesized and secreted by retinal pigment epithelial cells and its expression is upregulated by vitamin A.\textsuperscript{7,8} Recent studies also suggest an important role for TSP1 in choroidal vascular homeostasis and the pathogenesis of CNV.\textsuperscript{9} Immunohistochemical analysis of sections prepared from human eyes indicated that TSP1 is present in the macula region of the Bruch membrane, choroidal capillaries, and larger choroidal vessels. In addition, a significant decrease in the level of TSP1 was detected in the Bruch membrane and choroidal capillaries from AMD eyes.\textsuperscript{10} We, therefore, hypothesized that TSP1 deficiency will exacerbate the pathogenesis of CNV and it may be an important target for inhibition of CNV. Herein, we investigated the impact of TSP1 deficiency on laser-induced CNV using TSP1−/− mice. We also determined the potential therapeutic role of TSP1 in treatment of CNV using a TSP1 antiangiogenic mimetic peptide.

**METHODS**

**ANIMALS**

All animal studies were conducted in accordance with an animal protocol reviewed and approved by the University of Wisconsin–Madison Animal Care and Use Committee and in accordance with the Association for Research in Vision and Ophthalmic Statement for the Use of Animals in Ophthalmic and Vision Research. Six-week-old female wild-type (TSP1+/+) or TSP1-deficient (TSP1−/−) C57BL/6j mice (10 per group) were used in these studies and housed on a 12-hour light-dark cycle, with food and water provided ad libitum. Laser photocoagulation (75-µm spot size; 0.1-second duration; 120 mW) was performed in the 9-, 12-, and 3-o’clock positions of the posterior pole of each eye with the slitlamp delivery system of an OcuLight GL diode laser (Iridex) and a handheld coverslip as a contact lens to view the retina. After 14 days, the eyes were removed and fixed in paraformaldehyde, 4%, at 4°C for 2 hours. Following 3 washes in phosphate-buffered saline (PBS), the eyes were sectioned at the equator, and the anterior half, vitreous, and retina were removed. The remaining eye tissue was incubated in blocking buffer (20% fetal calf serum and 20% normal goat serum in PBS) for 1 hour at room temperature, followed by incubation with anti–intercellular adhesion molecule 2 (1:500 in PBS containing 20% fetal calf serum and 20% normal goat serum; BD Pharmagen) overnight at 4°C. The remaining eye tissue was then washed 3 times with PBS and incubated with the appropriate secondary antibody. The retinal pigment epithelium–choroid-sclera complex was dissected through 5 to 6 relaxing radial incisions and flat mounted on a slide with VectaMount AQ (Vector Laboratories). The samples were viewed by fluorescence microscopy and images were captured in digital format using a Zeiss microscope (Zeiss). ImageJ software (National Institute of Mental Health; http://rsb.info.nih.gov/ij/) was used to measure the total area (in micrometers squared) of CNV associated with each burn.

**IMMUNOHISTOCHEMISTRY FOR DETECTION OF MACROPHAGES**

Eyes were enucleated 3 days after laser injury, when the maximum number of macrophages were observed,\textsuperscript{11} and fixed in paraformaldehyde, 4%, for 2 hours, and retinal pigment epithelium–choroid-sclera complexes were dissected. Flat mounts were treated with blocking solution (20% fetal calf serum and 20% normal goat serum in PBS) for 1 hour at room temperature, followed by incubation with anti–F4/80 (1:500 in PBS containing 20% fetal calf serum and 20% normal goat serum; BD Pharmagen) overnight at 4°C. The remaining eye tissue was then washed 3 times with PBS and incubated with the appropriate secondary antibody. The tissue was viewed by fluorescence microscopy and images were captured in digital format using a Zeiss microscope. For quantitative analysis of macrophage recruitment, the area of fluorescence and average pixel intensities (mean gray value) were determined using Photoshop software (Adobe). The integrated density was calculated by multiplying the area of fluorescence by the mean gray value.

**TSP1 ANTIANGIOGENIC MIMETIC PEPTIDE TREATMENT**

The antiangiogenic activity of TSP1 is mapped to peptides from type I repeats and the procollagen homology domain.\textsuperscript{12} An overlapping peptide that expands these regions has shown good efficacy for inhibition of angiogenesis in various tumor models\textsuperscript{13} and was the basis for development of ABT510 and most recently a newer generation,\textsuperscript{14} ABT898. The amino acid sequence of ABT898 is N-acetyl-glycine-valine-D-alloisoleucine-serine-glutamine-isoleucine-arginine-prolin-ethylamid and was synthesized at the University of Wisconsin Biotechnology Peptide Synthesis core facility. The purity and sequence of the peptide were confirmed using standard methods. The peptide was dissolved in dextran solution, 5%, and used for intravitreal injections. The TSP1+/+ mice and TSP1−/− mice were injected intravitreally with the TSP1 antiangiogenic mimetic peptide (2 µL of 100 µg/mL) at day 0, right after laser rupture of the Bruch membrane. This dose was found to be most effective. Intravitreal injections were performed with a pump microinjection...
Thrombospondin 1 is a potent inhibitor of angiogenesis and can directly act on endothelial cells, inhibiting their proliferation and migration and promoting their apoptosis. The impact of TSP1 on choroidal endothelial cells, and more specifically on CNV, remains elusive. To determine whether lack of TSP1 expression would impact the degree of neovascularization on laser-induced CNV, and more specifically on CNV, remains elusive. We next determined whether administration of the TSP1 mimetic antiangiogenic peptide can inhibit CNV and/or reduce the area of neovascularization in TSP1−/− mice. Peptides derived from TSP1 type 1 repeats and the procollagen homology domain, including the peptide used herein, can signal through CD36 (TSP1 receptor) inhibiting angiogenesis.29,30 Recent studies have demonstrated that this may also require interaction of TSP1 with CD47 (receptor for C-terminal domain of TSP1).31 The TSP1 antiangiogenic mimetic peptide was administered intravitreally to TSP1−/− mice subjected to the laser-induced CNV. We observed a significant decrease in the mean (SD) area of CNV in TSP1−/− mice that received the TSP1 mimetic peptide compared with TSP1+/+ mice with peptide: 2254 [1040] µm² vs TSP1−/− mice with vehicle: 11340 [3315] µm²; n=15). Thus, enhanced CNV is observed in the absence of TSP1.

**STATISTICAL ANALYSIS**

Statistical differences between groups were evaluated with the unpaired t test (2-tailed). Means and standard deviations are shown. P≤ .05 is considered significant.

**RESULTS**

**TSP1 DEFICIENCY RESULTS IN INCREASED AREAS OF CNV**

Our results in Figure 1 and Figure 2 coupled with studies demonstrating decreased levels of TSP1 in choroidal membranes prepared from eyes with CNV18,19 suggest that TSP1 may suppress CNV. We next determined whether administration of the TSP1 mimetic antiangiogenic peptide can inhibit CNV and/or reduce the area of neovascularization in TSP1−/− mice. Peptides derived from TSP1 type 1 repeats and the procollagen homology domain, including the peptide used herein, can signal through CD36 (TSP1 receptor) inhibiting angiogenesis.29,30 Recent studies have demonstrated that this may also require interaction of TSP1 with CD47 (receptor for C-terminal domain of TSP1).31 The TSP1 antiangiogenic mimetic peptide was administered intravitreally to TSP1−/− mice subjected to the laser-induced CNV. We observed a significant decrease in the mean (SD) area of CNV in TSP1−/− mice that received the TSP1 mimetic peptide compared with vehicle control (TSP1−/− mice with peptide: 2254 [1040] µm² vs TSP1−/− mice with vehicle: 11340 [3315] µm²; n=15) (Figure 3A and B). The mean CNV area in eyes treated with the TSP1 peptide was decreased by approximately 80% compared to vehicle.
with that seen in eyes receiving vehicle alone (Figure 3C) \((P<.05)\). Administration of the TSP1 peptide to TSP1+/+ mice also significantly inhibited CNV lesion formation (mean \([SD]\), TSP1+/+ mice with peptide: 1840 [379] \(\mu\)m² vs TSP1+/+ mice with vehicle: 3235 [910] \(\mu\)m²; \(n=15\)) (Figure 4) \((P<.05)\). Thus, the TSP1 antiangiogenic mimetic peptide may provide an alternative treatment for CNV.

The studies presented herein demonstrate that lack of TSP1 results in enhanced CNV, and this was associated with a significant increase in the number of macrophages recruited to the sites of laser lesions. These observations are consistent with the important role of inflammation processes in the pathogenesis of CNV and the ocular anti-inflammatory role previously demonstrated for TSP1. In addition, we showed that administration of the TSP1 antiangiogenic mimetic peptide inhibited CNV in both TSP1+/+ and TSP1−/− mice. Thus, modulation of TSP1 expression or its antiangiogenic mimetic peptides may provide a novel approach for the treatment of CNV associated with AMD.

Histological studies have demonstrated the immediate arrival of macrophages at laser rupture sites within 1 hour of laser application, and macrophages accumulate in areas of disruption of the Bruch membrane \(^{24,32,34}\). Laser-induced CNV is an appropriate model for investigating the relationship between inflammation and angiogenesis. Macrophages secrete interleukin 1\(\beta\), among other inflammatory cytokines, in response to tissue injury and inflammation and promote angiogenesis \(^{33,34}\).

There has been considerable discussion of the role of inflammation in promoting ocular angiogenesis, particularly in neovascular AMD. Inflammation is critically involved in the formation of CNV lesions and contributes to the pathogenesis of AMD. Inflammatory cells are found in surgically excised CNV lesions from patients with AMD and in autopsied eyes with CNV. In particular, macrophages have been implicated in the pathogenesis of AMD because of their spatiotemporal distribution in the proximity of the CNV lesions in experimental models and humans, making significant contribution to the pathogenesis of CNV \(^{24,32}\).

The majority of the macrophages found in the proximity of the laser-induced CNV lesions are derived from newly recruited peripheral blood monocytes and are not
resident macrophages.\textsuperscript{34,35} Because macrophages play such a critical role in CNV formation, prevention of monocyte recruitment and infiltration into ocular tissues may ameliorate the development of CNV, a function that could be exploited therapeutically.\textsuperscript{34,32,36,37} Herein we showed that lack of TSP1 exaggerates CNV and this was associated with increased recruitment of macrophages into the sites of lesions. These results are consistent with previous reports of impaired TSP1 expression in the Bruch membrane and choroidal vessels of eyes with AMD\textsuperscript{38,39} and identification of TSP1 as a genetic loci that controls the size of CNV.\textsuperscript{39}

The anti-inflammatory nature of the intraocular environment is critical to the immune privilege of the eye and pathogenesis of CNV. Investigation into the role of inflammmation in neovascular eye disease often overlooks the principles of immune privilege in the eye. The ocular environment is generally not proinflammatory and it prohibits inflammation at the expense of certain immune effector mechanisms. It has been suggested that the loss of immune privilege as the eye ages may contribute to the increases in neovascular disease.\textsuperscript{40,41} These concepts challenge the idea that neovascular disease is simply an inflammatory process and support the idea that these diseases may result from the loss or dysfunction of important components of the cellular immune system.\textsuperscript{24}

Inflammatory mechanisms and immune activation have been implicated in the pathogenesis of CNV. Retinal laser burns disrupt the immune privilege in the eye resulting in inflammation.\textsuperscript{42,43} In addition, TSP1 plays a vital role in the modulation of immune privilege such that in its absence the retinal microenvironment is more proinflammatory and supports an activated state of microglia with poor recovery from injury.\textsuperscript{42} This is consistent with the increased recruitment of macrophages and enhanced severity of neovascularization observed herein in TSP1\textsuperscript{−/−} mice. The anti-inflammatory activity of TSP1 in the eye is mainly attributed to the enhanced levels of active transforming growth factor β (TGF-β) in the presence of TSP1.\textsuperscript{44,45} A major physiological function of TSP1 is activation of latent TGF-β with important function during normal developmental processes.\textsuperscript{46} The TSP1 anti-

angiogenic peptide used herein lacks the TSP1 sequence that is responsible for activation of TGF-β. Thus, the anti-inflammatory activity observed herein may be independent of the TSP1 effects on activation of TGF-β and its anti-inflammatory effects on CNV. Thus, the intact TSP1 molecule may impact CNV through both its anti-inflammatory and antiangiogenic activities. The potential synergistic and/or additive effects of these TSP1 activities on CNV associated with AMD are the subject of current investigation in our laboratory.

In summary, TSP1-deficient mice exhibited significantly enhanced choroidal neovascular membrane formation associated with increased inflammation and recruitment of macrophages. Together these results suggest that modulation of TSP1 plays an important role in CNV associated with AMD. Furthermore, TSP1 mimetic peptides could be used as novel therapeutics to inhibit CNV perhaps by modulating both the inflammatory and angiogenic states of choroidal vessels.

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