Endogenous Osteopontin Involvement in Laser-Induced Choroidal Neovascularization in Mice

Noribito Fujita, Shuko Fujita, Naboko Ogata, Masato Matsuoka, Yuka Okada, Shigeyuki Kon, Tosbimitsu Uede, and Shizuya Saika

PURPOSE. To evaluate the effects of the lack of osteopontin (OPN) and the administration of anti-OPN antibody on inflammation and vascular endothelial growth factor (VEGF) expression in choroidal tissue and on the development of choroidal neovascularization (CNV) after retinal photocoagulation in mice.

METHODS. CNV was induced in one eye each of 20 C57BL/6-background OPN-deficient mice or 20 wild-type littermates. In another series of experiments, CNV was induced in 40 C57BL/6 mice treated with intraperitoneal administration of 400 μg anti-OPN (SLAYGLR) neutralizing antibody or control IgG. Four laser spots were prepared in each eye. At day 14, the size of the CNV was evaluated by high-resolution angiography with fluorescein isothiocyanate (FITC)-dextran. Six wild-type or six knockout mice also received photocoagulation and processed for histology. mRNA expression of OPN, VEGF, and F4/80 macrophage antigen in laser-irradiated choroidal tissues was analyzed at day 3 in wild-type or knockout mice as well as in wild-type mice treated with anti-OPN antibody or control antibody.

RESULTS. Photocoagulation upregulated OPN expression in choroidal tissue. Histology did not uncover the effects of the lack of OPN on the heating of laser injury in choroid. The lack of OPN or systemic administration of anti-OPN antibody suppressed mRNA expression of VEGF and macrophage invasion in choroidal tissue. FITC-dextran angiography showed that lacking OPN or systemic anti-OPN antibody reduced the size of laser-induced CNV.

CONCLUSIONS. OPN is upregulated in laser-irradiated choroidal tissue. Endogenous OPN is required for macrophage inflammation and VEGF expression in choroidal tissue and for CNV development after retinal photocoagulation in mice. (Invest Ophthalmol Vis Sci. 2011;52:9310–9315) DOI:10.1167/iovs.10-7050

Choroidal neovascularization (CNV) is a major feature of the exudative form of age-related macular degeneration (AMD). In CNV lesions, new blood vessels grow from the choroid and penetrate Bruch’s membrane, invading the subretinal space.1 Experimentally, by using laser photocoagulation of the ocular fundus, CNV that models AMD is induced by the breakdown of Bruch’s membrane and the induction of local inflammation. CNV can induce retinal pigment epithelium detachment or serous retinal detachment; in the later phase, fibrovascular tissue is formed around CNV. All these lesions account for impaired vision in patients with AMD. Although the exact mechanism of the development of CNV in AMD patients and in experimental animals remains to be clarified, the major proangiogenic factor, vascular endothelial growth factor (VEGF), is believed to be involved in its pathogenesis.2–6 VEGF is expressed by various cell types, including macrophages, that are reportedly involved in the development of CNV in human AMD or in animal CNV.7,8

Investigations on non-VEGF components that are involved in the development of CNV have been reported. For example, tumor necrosis factor exhibits positive and negative roles in the growth of CNV, depending on the expression pattern of its receptors.9 Targeting a cytoskeletal component or an enzyme involved in extracellular matrix maturation, lysyl oxidase, reportedly suppresses the development of CNV.10,11

Osteopontin (OPN) is a secreted noncollagenous, sialic acid-rich protein that plays important roles in modulating cell behavior. It also functions as a cytokine that regulates the activities of tissue cells (epithelial cell types and mesenchymal cells), macrophages, and other immune cells at sites of injury.12–23 The thrombin-cleavage cryptic terminal—SVVYGLR in human OPN or SLAYGLR in mice—is capable of binding to α9 integrin and exhibits such cytokine-like roles of proinflammatory or proangiogenic effects.24–26 OPN function in tissue repair has been investigated.27–29 It has been reported that the lack of OPN perturbs repair processes of the cutaneous connective tissue or stromal healing in the cornea.30,31 OPN is also reportedly required for the development of neovascularization in bone formation or cancer progression.32–36 We previously reported that lacking OPN impairs cauterization-induced neovascularization in a mouse cornea. In this case, we showed that the loss of OPN in mouse ocular fibroblasts attenuated their angiogenic gene expression. AMD is also a neovascularization-related ocular disease; the development of neovascularization in choriotelial tissue in an AMD patient severely impairs the patient’s vision. Intravitreal injection of anti-VEGF antibody exhibits a therapeutic effect in suppressing the growth of CNV. Although OPN is known to be involved in the formation of neovascular tissue in other organs, it is still to be determined whether OPN is involved in the development of CNV and whether the lack of OPN or the administration of anti-OPN neutralizing antibody inhibits the development of experimental CNV in animals (i.e., mice). We induced CNV lesions using an argon laser photocoagulator in OPN-deficient mice or wild-
type (WT) mice treated with anti-OPN neutralizing antibody. Our data demonstrate a significant decrease in the size of experimental CNV lesions in OPN-null (knockout [KO]) mice or in mice treated with anti-OPN antibody.

**Materials and Methods**

Experiments were approved by the DNA Recombination Experiment Committee and the Animal Care and Use Committee of Wakayama Medical University. They were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Expression Pattern of VEGF, F4/80, and OPN in Argon Laser–Irradiated Choroidal Tissue**

Fifty-four C57BL/6 mice (WT) were included, and one eye of each was used. Retinas received laser photocoagulation for the induction of experimental CNV under slit lamp microscope observation\(^1\) and an argon laser photocoagulator (Lambda Plus; Coherent Inc., Santa Clara, CA). A coverslip was applied to the cornea to view the retina with sodium hyaluronate. Four lesions were produced using a power of 200 mW, a spot size of 50 μm, and a duration of 100 ms. Lesions were located at the 3-, 6-, 9-, and 12-o’clock meridians centered on the optic nerve and were located two or three disc diameters from the optic nerve. At days 1 (16 eyes) and 3 (20 eyes) and immediately after laser irradiation (time 0, 18 eyes), the mice were killed by CO\(_2\) asphyxiation. Each eye was enucleated. The cornea, iris, lens, and retina were removed from the sclera of the enucleated eye, and the choroidal tissue was obtained.

Because the upregulation of OPN mRNA expression was observed at day 3, as described, immunohistochemical detection of OPN protein in choroidal tissue was performed 3 days after laser treatment. Four eyes of four WT mice underwent photocoagulation, as described; 3 days later the mice were killed and their eyes were enucleated. After fixation in 4% paraformaldehyde, the specimens and the uninjured control eyes were embedded in paraffin. Sections were cut and processed for immunofluorescent staining for OPN by using a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). A coverslip was applied to the cornea to view the retina with sodium hyaluronate. Four lesions were produced using a power of 200 mW, a spot size of 50 μm, and a duration of 100 ms. Lesions were located at the 3-, 6-, 9-, and 12-o’clock meridians centered on the optic nerve and were located two or three disc diameters from the optic nerve. At days 1 (16 eyes) and 3 (20 eyes) and immediately after laser irradiation (time 0, 18 eyes), the mice were killed by CO\(_2\) asphyxiation. Each eye was enucleated. The cornea, iris, lens, and retina were removed from the sclera of the enucleated eye, and the choroidal tissue was obtained.

Because the upregulation of OPN mRNA expression was observed at day 3, as described, immunohistochemical detection of OPN protein in choroidal tissue was performed 3 days after laser treatment. Four eyes of four WT mice underwent photocoagulation, as described; 3 days later the mice were killed and their eyes were enucleated. After fixation in 4% paraformaldehyde, the specimens and the uninjured control eyes were embedded in paraffin. Sections were cut and processed for immunofluorescent staining for OPN by using a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

For the purpose of understanding the effects of lacking or blocking OPN on angiogenic gene expression and inflammation, we performed gene expression analysis in the C57BL/6-background KO mice. We subjected one eye each of 20 adult WT or 20 KO mice to laser irradiation, 20 WT mice to systemic administration of control antibody, and 20 WT mice to treatment with intraperitoneal anti-OPN antibody, as described. Three days after laser treatment, the mice were euthanized and the choroidal tissue was obtained as described. Choroidal tissues from two eyes were merged into one RNA sample; thus, we had 10 RNA samples in each experimental condition, as previously reported.\(^{37,38}\) The expression level of OPN was assayed with real-time RT-PCR (TaqMan; Propriety Gene Expression Assays, Applied Biosystems, Foster City, CA). All the data were normalized for endogenous expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mm03394295) by using ΔΔC\(_t\), and were statistically analyzed with the Tukey-Kramer test.

**Experimental CNV Model in KO Mice**

As described here in detail, real-time RT-PCR showed that OPN expression was upregulated in the photocoagulated choroidal tissue, and the loss of OPN suppressed the expression of VEGF and macrophage invasion in the tissue. These data prompted us to hypothesize that lacking or blocking OPN might affect the growth of experimental CNV. We therefore first examined whether lacking OPN might affect the growth of experimental laser-induced CNV in mice. Twenty-six age- and sex-matched KO mice and 26 control WT littermates were used. One eye was used in each mouse. Retinas underwent laser photocoagulation for the induction of experimental CNV under slit lamp microscopy.\(^1\) Fourteen days after laser photocoagulation, all 80 laser spots were used for fluorescein-dextran angiography to quantify the size of the CNV lesion in each of 20 WT or 20 KO mice. Laser-irradiated eyes in each genotype of mice were processed for histology by preparing cryosections (two WT and two KO mice) or paraffin sections (four WT and four KO mice).

**Experimental CNV Model in Mice Treated with Anti-OPN Neutralizing Antibody**

Adult male C57/BL/6 mice received intraperitoneal injections of anti-OPN neutralizing antibody, which reacts with the thrombin-cleavage terminal of the SLAYGLR sequence of mouse OPN (400
µg/mouse; 20 mice) or control nonimmune IgG (400 µg/mouse; 20 mice) at 2 and 5 days after laser photocoagulation in one eye according to the protocol described. All 80 laser irradiation spots in each WT or KO group were used to quantify the size of the CNV lesion.

Quantitation of the Size of CNV Lesions

Histology does not provide a quantitative evaluation of the CNV lesion. Thus, we performed the following assay of the size of CNV by using choroidal flat mounts. Fourteen days after laser photocoagulation, mice were anesthetized and perfused through the left ventricle with a 2% paraformaldehyde solution, 100 µL of 50 mg/mL FITC-labeled dextran in 10% gelatin (2 × 10^6 MW; Sigma, St. Louis, MO). The mice were killed by CO₂ asphyxiation. The eyes were enucleated and fixed in 4% paraformaldehyde for 24 hours. The anterior segment, crystalline lens, and retina were removed from the eyeball. The remaining sclera, with choroid and retinal pigment epithelium, was flat-mounted after several relaxing radial incisions and cover-glassed. Specimens were analyzed by fluorescence microscopy (U-CMAD3; Olympus, Tokyo, Japan) in a masked fashion by a research assistant. WinROOF software (Mitani Corporation, Tokyo, Japan) was used to measure the size of the hyperfluorescent areas corresponding to CNV.

Statistical Analysis in FITC-Dextran Angiography

A two-sample Student’s t-test with unequal variance was used for statistical analyses of the quantitative CNV flat-mount data. The hyperfluorescent areas of CNV lesions in each eye were measured, and the total area of CNV lesions per eye was determined as their sum. For confluent lesions, the entire hyperfluorescent area was measured, and the number of initial CNV lesions within confluent lesions was counted.

RESULTS

Expression Pattern of VEGF, F4/80, and OPN in Argon Laser–Irradiated Choroidal Tissue

In a WT mouse, mRNA expression of VEGF and F4/80 was significantly upregulated at day 3 or at days 1 and 5 after laser treatment, respectively (Figs. 1a, 1b). Expression of OPN was also upregulated at day 3, and OPN protein was readily observed immunohistochemically in the cells in the laser lesion of the choroidal tissue (Figs. 1c, 1d). We then examined whether such upregulation of OPN might affect the expression level of macrophage invasion and OPN by using an OPN-deficient mouse or systemic administration of anti-OPN neutralizing antibody. The loss of OPN suppressed VEGF expression and macrophage invasion (Fig. 3). Blocking OPN with systemic anti-OPN antibody did not affect mRNA expression of OPN (Fig. 3a) but inhibited VEGF expression and macrophage invasion (Figs. 3b, 3c).

Experimental CNV Model in KO Mice

Argon laser irradiation successfully induced CNV in a WT mouse. Hematoxylin and cosin histology showed the presence of scar-like choroidal tissue beneath the damaged retina at the site of laser injury, but no marked morphologic difference was observed (Fig. 4). To quantify the degree of CNV formation in mice, we evaluated the size of CNV lesions by FITC-dextran perfusion angiography. FITC-dextran perfusion was performed before choroidal flat mounts were prepared on day 14 after laser photocoagulation. The size of FITC-labeled CNV outgrowths was smaller in the KO group (80 spots in 20 eyes) than in the WT group (80 spots in 20 eyes) (Fig. 5).
Effects of Systemic Anti-OPN Antibody on Development of Laser-Induced CNV in Mice

We then examined, with the systemic administration of anti-OPN (SLAYGLR) antibody, whether blocking OPN effects represents the phenotype of the KO mouse in terms of laser-induced CNV development. Argon laser irradiation successfully induced CNV in WT mice that received anti-OPN antibody or control IgG. To quantify the degree of CNV formation in mice, we then evaluated the size of CNV lesions by FITC-dextran perfusion angiography. FITC-dextran perfusion was performed before choroidal flat mounts were prepared on day 14 after laser photocoagulation. The size of FITC-labeled CNV outgrowths was smaller in an anti-OPN antibody group (80 spots in 20 eyes) than in a control IgG group (80 spots in 20 eyes) (Fig. 6).

**DISCUSSION**

Our data demonstrated first that argon laser irradiation upregulates OPN mRNA expression along with VEGF upregulation and induction of macrophage (F4/80-positive) infiltration. Protein of OPN was also readily detected in the cells in the laser lesion of the choroid. These findings prompted us to hypothesize that OPN might be involved in the development of laser-induced experimental CNV in an animal. As for the laser irradiation-induced CNV model, the breakdown of the Bruch’s membrane and laser-induced local inflammation might be involved in the development of neovascular tissue by the inflammation-related upregulation of angiogenic cytokines (i.e., VEGF). To explore this hypothesis, we took advantage of the availability of a mouse lacking OPN and of neutralizing antibodies against the human or mouse thrombin-cleavage terminal of OPN that exhibits proangiogenic or proinflammatory activity. In the in vivo experiment, we first examined the level of angiogenic gene expression in the absence of OPN activity. Real-time RT-PCR showed that the loss of OPN or the systemic administration of anti-OPN antibody suppressed the expression level of F4/80 macrophage antigen and VEGF in the healing choroidal tissue after laser irradiation. Reduced infiltration of macrophages might account for the reduction in VEGF level in the tissue under the loss or blocking of osteopontin activity. These data strongly suggested OPN might be involved in the growth of experimental CNV. However, histology with hema-
toxylin and staining did not show the difference of the laser lesion in the choroidal tissue. CNV induced by laser irradiation was detected by CD31 immunohistochemistry (data not shown). We observed by histology that the development of the whole eye structure, including the choroid, was not affected by the loss of OPN (data not shown). However, this method was also considered not to be suitable for the comparison of the size of the CNV. We then showed, with FITC-labeled dextran angiography in flat mounted retinal specimens, that the lack of OPN reduced the size of experimental laser-induced CNV in mice. The effect of lacking OPN on the development of CNV was reproduced by systemic intraperitoneal administration of anti-OPN neutralizing antibody. These in vivo findings suggest that blocking OPN activity might be a potential strategy for the prevention/treatment of CNV development in an AMD patient.

Reports have been published indicating that lacking or blocking OPN results in the suppression of tissue local inflammation. A study from another laboratory also showed that OPN positively modulates the expression of VEGF and promotes neovascularization-dependent tumor growth. As for nonneoplastic tissues, OPN is reportedly involved in tissue inflammation in various tissues; it was reported that the loss of OPN or the systemic administration of anti-OPN antibody exhibits a therapeutic effect on experimental autoimmune arthritis, airway inflammation, and inflammatory fibrosis in the kidney and the spinal cord. In these experimental disease models, blocking OPN activity suppresses pathologic inflammation. In the eye, the loss of OPN reduces the severity of experimental uveitis in mice.

In addition to the OPN effect on local tissue inflammation and subsequent local expression level of angiogenic cytokines, OPN reportedly affects the behavior of vascular endothelial cells. The effects of OPN on in vitro neovascularization by cultured vascular endothelial cells and cell signaling were investigated. We observed that human umbilical vein endothelial cells cultured on a fibroblast feeder layer form a vessel-like tube structure when treated with the addition of an OPN peptide and that VEGF-promoted tube structure formation by the cells was blocked by the addition of anti-OPN antibody (Fujita N et al., unpublished data, 2011). Dai et al. also reported that OPN promotes vessel-like tube formation by vascular endothelial cells in association with the activation of phosphoinositide 3-kinase/Akt and nuclear factor-kB (NF-kB). We previously reported that the loss of OPN attenuates the phosphorylation of Smad2 and p38 mitogen-activated protein kinase on exposure of transforming growth factor β1 (TGF-β1) in cultured ocular fibroblasts. TGF-β is also reportedly involved in the growth of neovascular tissues. Although the exact mechanism of the suppression of TGF-β-related signals is to be investigated, the binding of OPN to its specific integrin receptors might support the full activation of growth factor–related signaling. Our examination in cultured ocular fibroblasts obtained from an eye of a postnatal day 1 mouse detected no influence of lacking OPN on the activation of p65 RelA of NF-kB (Fujita N et al., unpublished data, 2011).

OPN and tenasin C are both ligands of α9 integrin. We previously reported that blocking α9 integrin by using systemic administration of a neutralizing antibody suppresses experimental autoimmune arthritis in mice. It is to be examined whether the administration of an anti-α9 integrin antibody blocks the development of laser-induced CNV in animals.

In summary, our data provide evidence that endogenous OPN at physiological levels inhibits induced angiogenesis in laser-induced CNV in vivo. The mechanism of action might include the effects of OPN on vascular endothelial cells. Blocking OPN activity might be a potential strategy for the prevention or treatment of CNV in an AMD patient. A clinical trial using anti-OPN antibody is to be set up to establish a new AMD treatment strategy.

References


