Dose-Dependent Effect of Pitavastatin on VEGF and Angiogenesis in a Mouse Model of Choroidal Neovascularization

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Purpose. To evaluate the relation between statin therapy, vascular endothelial growth factor (VEGF) expression, vascular leakage, and CNV size in experimentally induced choroidal neovascularization (CNV).

Methods. Wild-type (C57 Bl/6) mice received pitavastatin 0.18 mg/kg per day (group 1), 1.8 mg/kg per day (group 2) or 18 mg/kg per day (group 3) for 3 days before laser-induced CNV and continued to receive the drug for 14 days. Serum total cholesterol levels were measured by spectrophotometry. Fluorescein angiograms were graded by masked observers. VEGF protein levels from retinal lysates were measured and CNV area was assessed by histology.

Results. Pitavastatin did not reduce total serum cholesterol at any of the doses used. The incidence rate ratios for development of clinically significant CNV leakage was 0.62 (95% CI, 0.46–0.84) for group 1, 0.56 (95% CI, 0.28–1.10) for group 2, and 1.22 (95% CI, 1.01–1.48) for group 3 (P = 0.002, 0.09, and 0.04, respectively). Mean CNV area increased by 13%, 22%, and 95% in groups 1, 2, and 3, respectively (P > 0.05). Normalized VEGF levels did not mirror the observed changes in fluorescein leakage and CNV area in histologic examination.

Conclusions. Pitavastatin therapy for experimental CNV in wild-type mice resulted in reduced fluorescein leakage at a dose of 0.18 mg/kg per day. The higher dose of 18 mg/kg per day resulted in increased fluorescein leakage and a trend toward an increase in CNV size, indicating a potentiating effect in choroidal neovascular disease. (Invest Ophthalmol Vis Sci. 2006;47:2623–2631) DOI:10.1167/iovs.05-0855

Visual loss in age-related macular degeneration (AMD) is predominantly associated with the development of choroidal neovascularization (CNV).1 An essential element in the development of CNV is the rupture of Bruch’s membrane and the proliferation of blood vessels through breaks in the membrane. Experimental CNV can be created by laser-induced rupture of Bruch’s membrane, which stimulates preexisting capillaries to proliferate into new capillary networks.2 There are a large number of specific proteins involved in angiogenesis that interact in complex ways, and their expression depends on the surrounding cell types. In the recent past, considerable attention has been directed to the lipid-lowering independent effects of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, generally called statins. The available data from population and case series investigations of the effect of statins on visual loss from exudative AMD is controversial.

Furthermore, initial reports by Kureishi et al.13 indicated that simvastatin promotes angiogenesis through the activation of Akt. The treatment of endothelial cells with L-mevalonate, the direct product of HMG-CoA reductase, inhibits the statin-induced Akt phosphorylation, implying that the observed effect is the direct consequence of HMG-CoA reductase inhibition.15 Subsequent reports from Vincent et al.14 demonstrated that another statin, cerivastatin, has antiangiogenic activity. Cerivastatin inhibits mitogen-induced endothelial migration. This effect is attributed to the inhibition of Rho A, which was shown to be mediated by the inhibition of focal adhesion kinase and Akt activation.15

The resulting controversy about the role of statins in angiogenesis has been addressed in three subsequent reports.16–18 Weis et al.18 were the first to demonstrate a dose-dependent biphasic effect of statins on angiogenesis, finding that low-dose cerivastatin (0.005–0.01 μM) increases cellular proliferation and cell migration distance, whereas higher concentrations (0.05 and 0.5 μM) exert the opposite effect. Similar effects on endothelial cell migration were shown with atorvastatin and mevastatin, but no inhibitory effects were observed with higher concentrations of statins on bone-marrow–derived endothelial progenitor cells indicating the importance of the cell milieu.17 Apoptosis was observed in both studies using the higher concentration of statins.17,18 Furthermore, Frick et al.16 demonstrated that simvastatin and atorvastatin increase vascular endothelial growth factor (VEGF) production at concentrations of 1 to 10 μM, but not at lower concentrations, in human umbilical endothelial cells (HUVECs) and decrease basal VEGF in human coronary vascular smooth muscle cells (HVSMSCs). The available in vitro data therefore suggest that statins promote angiogenesis at lower doses, and under certain conditions some statins, at least, have the opposite effect. The dose-dependent effect on angiogenesis did not correlate with VEGF levels.

Given the absence of in vivo data in a model of CNV to compare with the available in vitro data, the present study was designed to evaluate the effect of a novel statin, pitavastatin,19,20 on CNV and VEGF expression. The murine model of experimental laser-induced neovascular disease was used because the mouse is resistant to the hypcholesterolemic effect of statins.21,22

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METHODS

Experimental Groups
All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with protocols reviewed and approved by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Normal pigmented wild-type mice (C57BL/6J) aged 10 to 14 weeks were obtained from The Jackson Laboratory (Bar Harbor, ME). Pitavastatin (Livalo, previously known as NK-104) was kindly provided by Kowa Co. Ltd (Nagoya, Japan). Pitavastatin was dissolved in 0.5% carboxymethyl cellulose (CMC) sodium salt (Wako, Osaka, Japan) and administered at a dose of 0.18 mg/kg per day (group 1), 1.8 mg/kg per day (group 2), and 18 mg/kg per day (group 3). CMC 0.5% vehicle was administered to control mice. The dose of pitavastatin was based on the knowledge of the higher caloric requirements for the mouse (160 kcal/kg body weight0.75 per day) compared with humans (31 kcal/kg body weight per day)23. The requirement for calories is approximately 12 times higher in a 25-g mouse than in a 65-kg man. Given that the recommended dose of pitavastatin is 1 to 2 mg/d, the calculated mouse equivalent (dose used in group 1 mice) assuming an average mouse body weight of 20 g27 was 0.003–0.006 mg/d (0.18 – 0.36 mg/kg per day). Oral gavage was performed with a 20-gauge blunt feeding needle for 3 days before laser-induction of CNV and continued daily until fluorescein angiography was performed 14 days after laser-induction of CNV.

Induction of Choroidal Neovascular Membranes
Mice were anesthetized with a mixture of ketamine 100 mg/kg body weight (Abbott, North Chicago, IL) and xylazine 10 mg/kg body weight (Bayer, Humacao, Puerto Rico) by intraperitoneal injection. Pupils were dilated with topical 0.5% tropicamide, and CNV was induced with a diode-pumped frequency-doubled, 532-nm laser (Iridex; OcuLight GLx, Mountain View, CA), by using a modification of a previously described protocol.24 Four lesions were created with a power of 200 mW, a spot size of 50 μm, and a duration of 100 ms. The lesions were located at the 3, 6, 9, and 12 o’clock meridians centered on the optic nerve head and located approximately 2 to 3 disc diameters from the optic nerve head. The presence of a bubble at the time of lasering was taken as an indication of the rupture of Bruch’s membrane and injury sufficient to induce formation of CNV. We excluded eyes in which a massive subretinal hemorrhage developed after laser induction of CNV.

Fluorescein Angiography
The left eye of each mouse was used for the assessment of vascular leakage using fluorescein angiography (FA) according to our previously published protocol (control group, n = 6; group 1, n = 7; group 2, n = 6; group 3, n = 6).25 FA was performed in anesthetized animals with dilated pupils using a digital fundus camera (Model TRC 50 IA; Topcon, Paramus, NJ) and standard fluorescein filters. Fluorescein injections were administered by intraperitoneally (0.2 ml of 2% fluorescein; Akorn, Decatur, IL), and the timer was started as soon as the fluorescein bolus was injected. A PMMA contact lens (base curve, 1.65 mm, power 7.0 D, size 2.5 mm) was placed on the mouse cornea to improve visualization and prevent corneal drying (Unicon Corp., Osaka, Japan). After FA, the eyes were enucleated with the animals under deep anesthesia and the animals were euthanatized with a lethal dose of pentobarbital (Fetal-Plus solution; 390 mg/mL; Vortech Pharmaceuticals, Dearborn, MI).

Two masked retina specialists (ESG and JWM) not involved in lasering or angiography evaluated fluorescein angiograms obtained 14 days after laser-induction of CNV with angiographic standards. Lesions were graded based on our previously published protocol.25 Grade 0 lesions had no hyperfluorescence. Grade-1 lesions exhibited hyperfluorescence without leakage. Grade-2A lesions exhibited hyperfluorescence in the early or midtransit images and late leakage. Grade-2B lesions showed bright hyperfluorescence in the transit images and late leakage beyond the treated areas. Grade 2B lesions were defined as clinically significant).25

Histology
Three eyes of each group were enucleated 14 days after laser induction of CNV and fixed in 10% formalin. Hematoxylin-eosin serial 6-μm sections were stained, and three images were obtained per section to evaluate leakage.

Table 1. Lesions per Study Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice (n)</th>
<th>Lesions (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>28</td>
</tr>
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<td>2</td>
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<td>3</td>
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One eye per mouse was included in the study. Each eye had four CNV lesions induced by laser.

![Figure 1. The percentage of lesions graded as 0, 1, or 2A and lesions graded as 2B are shown for each group of mice.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932938/ on 06/16/2017)
sections were cut to determine the center of each lesion. Subsequent measurements were performed using stained sections examined at 20× magnification (model DM/RXA microscope; Leica, Wetzlar, Germany) with the observer masked to the treatment group. Images were digitized with a camera (Model ORCA ER; Hamamatsu, Hamamatsu City, Japan) and image analysis was performed on computer (OpenLab ver. 4.0.1; Improvision, Coventry, UK). The total CNV area was measured using the middle section of each lesion of each eye. CNV quantification was also estimated using the ratio (X/Y) of the maximum thickness from the bottom of the pigmented choroidal layer, to the top of the neovascular membrane (X), to the thickness of the intact pigmented choroid adjacent to the lesion (Y).

VEGF Enzyme-Linked Immunosorbent Assay

Eyes were enucleated 3 days after laser induction of CNV. The anterior segment and lens were removed, and the retina was carefully separated from the underlying choroid and sclera. The retina was sonicated in 100 μL mammalian cell lysis buffer (5× buffer Tris-EDTA, 5× NaCl, 5× lauryl sulfate, 5× deoxycholic acid, 5× Igepal CA 630, (protease

FIGURE 2. Early (a, c, e, g) and late (b, d, f, h) phase angiograms of mice retina 14 days after laser-CNV. Bold arrows: grade 2B CNV; block arrows: camera artifact (a, b, g, h). Representative angiograms for control mice show two of three CNV lesions with grade 2B leakage (bold arrows, a, b). In group 1 mice hyperfluorescence was observed at the site of laser injury without fluorescein leakage (c, arrows d); in group 2 mice one of four CNV lesions had grade 2B leakage (bold arrows, e, f), and for group 3 mice, three of four CNV lesions have grade 2B leakage (bold arrows, g, h).
inhibitor enzyme; Sigma-Aldrich, St. Louis, MO) and kept on ice for 30 minutes. The lysate was cleared of debris by centrifugation at 13,000 rpm for 30 minutes at 4°C. The supernatant was assayed for VEGF with an enzyme-linked immunosorbent assay (ELISA) kit (mouse VEGF Quantikine kit; R&D Minneapolis, MN). The choroid and sclera were prepared as a lysate in a similar way to the retina lysate preparation. Total protein was measured with a commercial assay (DC protein assay; Bio-Rad, Hercules, CA). Duplicate measurements were performed for all samples, standards, and controls.

To determine the effect of pitavastatin on VEGF formation in the posterior segment of the eye, we administered CMC or pitavastatin by oral gavage for 3 days without prior laser induction of CNV. VEGF measurements were performed in retina and choroid-sclera lysates.

### Cholesterol Measurements

Total cholesterol measurements were performed spectrophotometrically using the cholesterol/cholesterol ester quantitation kit (BioVision, Mountain View, CA) with blood from the tail vein at the start of the study (day 0) and by cardiac puncture in mice under deep anesthesia before death. Samples were measured in duplicate.
Statistical Analysis

Poisson regression analysis was performed to determine the likelihood of development of ‘clinically significant leakage,’ which we defined as grade-2B leakage at day 14 after laser induction of CNV. Incidence rate ratios (IRRs) were calculated to assess differences in the incidence rates of grade-2B CNV between treated and untreated mice. Given that the probability of development of significant leakage may be increased in CNV lesions originating from the same eye, we calculated the proportion of eyes with grade-2B CNV in each treatment group.

ANOVA was performed to analyze differences between the treatment groups and the vehicle control group for all other parameters evaluated in the study. For CNV area, the total CNV area of four lesions per eye was used for the analysis. For the X/Y height ratios of histology lesion height, the mean of four observations per eye were used for the analysis. Results are given as mean ± SE. \( P < 0.05 \) was considered significant.

RESULTS

Fluorescein Angiography

The number of mice examined by FA is shown in Table 1, and the percentages of lesions per eye demonstrating clinically significant leakage by FA are shown in Figure 1. The highest proportion of grade-2B lesions was found in group-3 mice (22/24 lesions), and the lowest proportion was found in group-2 mice (10/24 lesions; Fig. 1). The incidence rate ratios for developing clinically significant CNV leakage was 0.62 (95% CI, 0.46–0.84) for group 1, 0.56 (95% CI, 0.28–1.10) for group 2, and 1.22 (95% CI, 1.01–1.48) for group 3 (\( P = 0.002, 0.09, 0.04 \), respectively; Fig. 2).

Histology

We quantified the size of experimental CNV by determining the total CNV area for each eye and the X/Y ratio of the maximum thickness of each lesion to the thickness of the normal adjacent choroid, according to Lambert et al.\(^{26}\) Nonsignificant increases in mean total CNV area were observed in groups 1 to 3 compared with the vehicle control-treated mice. A minimal reduction in the X/Y ratio was observed for groups 1 and 2 compared with the vehicle-treated control mice, as well as a 35% increase in the X/Y ratio for group-3 mice (\( P > 0.05 \); Fig. 3).

VEGF Enzyme-Linked Immunosorbent Assay

ELISA of freshly isolated retinal lysates 3 days after laser-induced CNV demonstrated highest mean VEGF measurements in control mice treated with laser (58.97 ± 3.66 pg/mg total protein), and lowest in group-2 mice (47.23 ± 1.92 pg/mg total protein). The difference between the VEGF retinal lysate measurements was greatest between vehicle-treated control mice and group-2 mice (\( P = 0.15 \); Fig. 4a).

Mean basal VEGF levels in retinal lysates were 76% greater in vehicle-treated control mice that received laser treatment than in mice that did not undergo laser (\( P = 0.03 \), Wilcoxon rank sum test, figures 4a and 4b). Pitavastatin increased basal retinal lysate VEGF levels in the absence of laser-CN V by 135%, 75% and 58% in groups 1, 2 and 3 respectively; this finding was significant for group 1 only (\( P = 0.009 \), Fig. 4b).

ELISA of freshly isolated choroid/sclera lysates did not change significantly in any of the treatment groups after pitavastatin treatment (\( P < 0.05 \)). Two samples in group 3 had a protein level that was below the detectability level of the kit used. Normalized choroid/sclera lysate VEGF levels showed marked variability due to variability in the choroid–sclera lysate total protein levels.

Cholesterol Levels

Pitavastatin did not reduce total serum cholesterol at any of the doses used (Fig. 5). The mean total cholesterol was 163.3 ± 15.6 mg/dL in all mice at baseline. The mean total cholesterol before death was 125 ± 55.8 mg/dL (CMC group), 179.9 ± 10 mg/dL (group 1), 247.2 ± 28.3 mg/dL (group 2), and 176.5 ± 32.8 mg/dL (group 3), indicating an increase by 10%, 51%, and 8% in the three statin-treated groups, respectively. Levels of cholesterol were statistically significantly increased after statin treatment in group 2 mice only compared with the levels in the pretreatment group (\( P = 0.01 \)).
**DISCUSSION**

We present in this study the first in vivo evidence of a biphasic dose-dependent effect of statins in choroidal neovascular disease. Vascular leakage was decreased at the dose equivalent to the therapeutic dose of pitavastatin for human hypocholesterolemia, and increased significantly when the dose was increased by a factor of 100 independent of VEGF levels. Therefore, pitavastatin administered orally at 0.18 mg/kg per day in the mouse for 3 days before laser induction of CNV and continued for 2 weeks thereafter reduced the incidence of clinically significant vascular leakage in an experimental model of CNV, whereas the higher dose of 18 mg/kg per day increased the incidence of vascular leakage. These findings are consistent with histologic findings of smaller CNV size for the lower dose and larger CNV size for the higher dose. Retinal VEGF lysate levels 3 days after laser-induced CNV did not mirror the fluorescein and histology findings, suggesting a VEGF-independent mechanism for the observed statin effect. These findings were not associated with a reduction in serum cholesterol levels, indicating a mechanism that is independent of the cholesterol-lowering actions of pitavastatin.

Initial studies attributed the predominant beneficial effects of statins to their cholesterol-lowering properties. Several trials however, have subsequently demonstrated vascular protection independent of their lipid-lowering effects.
though AMD has been associated with markers of cardiovascular disease such as smoking, hypertension, and elevated serum cholesterol, one cannot make the direct inference that statins are therefore protective against AMD. Preliminary data suggest a protective effect of statins and cholesterol-lowering medications in AMD, but subsequent studies have shown inconsistent results. One study showed that subjects with CNV were significantly less likely to use statins than were subjects with dry AMD, suggesting a protective effect in neovascular disease. The suggested "protective" findings, however, were not consistent with the epidemiologic data of several population-based studies. Thus, in the Rotterdam study, subjects taking cholesterol-lowering medications had a similar incidence of AMD to those not using these medications and in the Beaver Dam Eye Study, statin use was not associated with the 5-year incidence of early or late AMD.

Vascular leakage and angiogenesis are two important factors that can lead to visual loss in neovascular AMD. Furthermore, there is both histologic and clinical evidence of an inflammatory component in neovascular macular degeneration. In vitro, statins inhibit leukocyte adhesion to endothelial cells and reduce cytokine production which controls leukocyte migration. Furthermore, lovastatin attenuates experimental autoimmune encephalomyelitis as well as experimental autoimmune uveitis through the inhibition of endothelial cell Rho-mediated lymphocyte migration. The antiatherosclerotic activities of statins have also been attributed, at least in part, to their anti-inflammatory effects. It is therefore possible that statins may attenuate experimental CNV formation in mice through a direct antiangiogenic effect, or possibly through an anti-inflammatory effect. Although VEGF is the major cytokine involved in angiogenesis and has been shown to be correlated with the amount of inflammatory cells in CNV from AMD patients indicating that inflammation plays an important role in CNV formation, our data do not support a role for VEGF as a mediator of the observed effect of statins on CNV. Pitavastatin also has antioxidant effects demonstrated by the activation of the transcription of the human serum paraoxonase 1 gene and by inhibiting generation of reactive oxygen species. Given the well-documented benefits of oral antioxidant therapy and zinc in macular degeneration, this may constitute a mechanism for the reduction in CNV activity.

Some of the beneficial effects of statins have been attributed to their proangiogenic properties. In a model of transient forebrain ischemia, pitavastatin at a dose of 10 mg/kg (a dose closer to that in group 3 in the present study) promotes angiogenesis by upregulating endothelial nitric oxide synthetase (eNOS). Evidence suggests that these protective effects may be mediated by the protein kinase Akt which regulates angiogenesis and promotes eNOS upregulation.

The present study yields further support to current prescribing patterns of statins, which have now become a cornerstone for the treatment of hyperlipidemia, given the observed biphasic effect and absence of deleterious effects for CNV leakage at clinical hypocholesterolemic doses. The complex molecular mechanisms for the observed pitavastatin effect on CNV were not studied in this investigation. In vitro data indicate that statins have a proangiogenic effect at lower concentrations and antiangiogenic effect at higher concentrations, but VEGF synthesis was shown to depend on the cell type under investigation and the observed effects on VEGF were not necessarily paralleled by the angiogenic activity of endothelial cells. A toxic effect at high doses of statins was manifested in our study by an increase in CNV leakage in group-3 mice.

Therefore, our data indicate that therapeutic doses of pitavastatin reduce the incidence of CNV formation, but increasing the dose 100-fold may exacerbate CNV leakage. A prospective randomized clinical trial may be warranted, but important considerations should include ethical considerations regarding placebo therapy in patients with cardiovascular disease, sample size, length of treatment, and potential costs involved.

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References


