Inhibition of Laser-Induced Choroidal Neovascularization by Atorvastatin by Downregulation of Monocyte Chemotactic Protein-1 Synthesis in Mice

Kiyoshi Yamada, Eiji Sakurai, Masahiro Itaya, Satoshi Yamasaki, and Yuichiro Ogura

PURPOSE. To determine the effect of atorvastatin, an HMG CoA reductase inhibitor, on experimental choroidal neovascularization (CNV) induced by laser photoagulation in mice.

METHODS. CNV was induced by laser photoagulation in normal wild-type mice. The mice received either oral atorvastatin 10 (AS10 group) or 20 (AS20 group) mg/kg per day for 3 weeks and before and after laser application; 1 (AS1) and 2 (AS2) mg/kg per day were included in the measurement of the parameters of CNV volume and the expression of chemokine ligand 2/monocyte chemotactic protein-1 (CCL2/MCP-1) and intracellular adhesion molecule (ICAM)-1. CNV responses were compared based on volume measurements 2 weeks after laser photoagulation. Expression of vascular endothelial growth factor (VEGF), CCL2/MCP-1, and ICAM-1 in the RPE and choroid was quantified by ELISA 2 or 3 days after photoagulation. Macrophage infiltration of the choroid was determined by flow cytometry.

RESULTS. The mean CNV volume was significantly smaller in the AS1 (44.16 ± 4.67 × 10³ μm³), AS2 (36.49 ± 4.64 × 10³ μm³), AS10 (25.75 ± 2.41 × 10³ μm³), and AS20 (33.24 ± 8.42 × 10³ μm³) groups compared with control mice (64.21 ± 10.73 × 10³ μm³; P = 0.0004, P < 0.0001, P < 0.0001, P < 0.0001, respectively). The mean VEGF and CCL2/MCP-1 protein levels decreased significantly (P = 0.001, P = 0.02, respectively) in the treated group compared with the control group. ICAM-1 expression did not differ significantly between the treated and control groups. The number of choroid-infiltrating macrophages decreased markedly in the treated group.

CONCLUSIONS. Atorvastatin effectively inhibited laser-induced CNV in mice and was associated with downregulation of CCL2/MCP-1 and VEGF and reduced macrophage infiltration into the RPE/choroid. (Invest Ophthalmol Vis Sci. 2007;48:1839–1843) DOI:10.1167/iovs.06-1085

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness among elderly individuals,1 yet little is known about the molecular mechanisms of choroidal neovascularization (CNV), the angiogenic process responsible for the most severe vision loss in patients with AMD.2 An inflammatory process may be a critical factor contributing to CNV development,3 and it is known that the relation of macrophages with chemokine ligand 2/monocyte chemotactic protein 1 (CCL2/MCP-1) is important in inflammation reaction.4,5 It has also been reported that intracellular adhesion molecule (ICAM)-1 is upregulated and promotes macrophage infiltration of the retinal pigment epithelium (RPE) in CNV.6 Current treatment options are limited to the late stage of the disease when central vision is already greatly threatened, and even new treatments have little impact on the rate of blindness.7

AMD has been associated with markers of atherosclerotic disease, such as carotid plaques,8 elevated pulse pressure,9 and risk factors for cardiovascular disease, including smoking,1,9 hypertension,1,10 and elevated serum and dietary cholesterol levels.11,12 Histologic evidence suggests that cholesterol accumulation in Bruch’s membrane may play a role in the pathogenesis of AMD.13 These data suggest that the causal pathways for cardiovascular disease and AMD may share similar risk factors.

Atorvastatin, an HMG CoA reductase inhibitor, has been largely used in patients with atherosclerotic disease and hyperlipidemia.14 The drug has pleiotropic benefits independent of cholesterol levels, including antioxidant and anti-inflammatory effects. The evidence has been reviewed and the rationale discussed behind recent suggestions that cholesterol-lowering agents may be useful to treat early AMD.15 There is growing evidence that leukocyte-mediated angiogenesis involves the interaction of cellular adhesion molecules and VEGF, and leukocyte-endothelial adhesion is a crucial step in inflammation or neovascularization and is implicated in the pathogenesis of CNV.16,17 Moreover, statins inhibit high-glucose-mediated neutrophil–endothelial cell adhesion through decreasing surface expression of endothelial adhesion molecules.18 Statins have been reported to have anti-inflammatory effects and inhibit leukocyte adhesion and migration, independent of the cholesterol-lowering effect.19,20

In the present study, we investigated whether a statin inhibits CNV in vivo and analyzed RPE macrophage infiltration and VEGF expression.

METHODS

Animals

Male wild-type C57BL/6 mice (Japan SLC, Shizuoka, Japan) between 6 and 8 weeks of age were used to minimize variability. For all procedures, anesthesia was induced by an intramuscular injection of 50 mg/kg ketamine HCl (Sankyo, Tokyo, Japan) and 10 mg/kg xylazine (Bayer, Tokyo, Japan), and the pupils were dilated with topical 1% tropicamide (Santen, Osaka, Japan). All animal experiments were performed in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Drug Treatments

Atorvastatin was supplied by Pfizer (Tokyo, Japan). The mice had free access to standard chow and tap water and received either oral ator-
vastatin 10 (AS10 group) or 20 (AS20 group) mg/kg per day in 0.5% methylcellulose by oral gavage once daily 3 days before and after laser application; 1 (AS1) and 2 (S2) mg/kg per day were included in the measurement of the parameters of CNV volume and expression of CCL2/MCP-1 and ICAM-1.

**Induction of CNV**

Laser photocoagulation (532 nm, 200 mW, 100 ms, 100 μm; Elite, Lumenis, Salt Lake City, UT) was performed (area studies: three spots/eye; protein analyses or flow cytometry: 25 spots/eye) bilaterally in each animal on day 0 by one individual masked to the drug group assignment. Laser spots were applied in a standard fashion around the optic nerve using a slit lamp delivery system and a coverslip as a contact lens. The morphologic end point of the laser injury was the appearance of a cavitation bubble, which is thought to correlate with the disruption of Bruch's membrane.

**Volume of CNV**

Two weeks after laser injury, the animals were euthanatized with a lethal dose of pentobarbital sodium (Abbott Laboratories, Lake Forest, IL), and the eyes were enucleated and fixed with 4% paraformaldehyde for 30 minutes at 4°C. Eye cups obtained by removing the anterior segments were washed three times in phosphate-buffered saline (PBS), followed by dehydration and rehydration through a methanol series. After blocking twice with buffer (PBS containing 1% bovine serum albumin [BSA]; Sigma-Aldrich, St. Louis, MO) and 0.5% Triton X-100 (Sigma-Aldrich) for 30 minutes at room temperature, the eye cups were incubated overnight at 4°C with 0.5% FITC-Griffonia simplicifolia isocolectin-B4 (Vector Laboratories, Burlingame, CA), which binds to terminal β-galactose residues on the surface of endothelial cells and selectively labels the murine vasculature. Diluted with PBS containing 0.2% BSA and 0.1% Triton X-100. After two washings with PBS containing 0.1% Triton X-100, the neurosensory retina was gently detached and severed from the optic nerve. Four relaxing radial incisions were made, and the remaining RPE/choroid-sclera complex was flattened in antifade medium (Immuno-Mount Vecstarchad Mounting Medium; Vector Laboratories) and coverslipped. Flatmounts were examined with a scanning laser confocal microscope (LSM 5 Pascal; Carl Zeiss Meditec, GmbH, Oberkochen, Germany). The vessels were visualized by excitation with a blue argon laser wavelength (488 nm) and the emissions detected in the RPE/choroid-sclera complex. The deepest focal plane in which the laser could be identified was judged to be the floor of the lesion. Images of each section were stored digitally. The area of CNV-related fluorescence was measured by computerized image analysis with the microscope software. The sum of the entire area of fluorescence in each horizontal section were stored digitally. The area of CNV-related fluorescence was measured by computerized image analysis with the microscope software.

**VEGF, CCL2/MCP-1, and ICAM-1 ELISA**

To detect VEGF protein levels in the RPE/choroid lysates, 3 days after application of 25 laser spots the eyes were enucleated, and the RPE/choroid complex was sonicated in lysis buffer (20 mM imidazole HCl, 10 mM KCl, 1 mM MgCl₂, 10 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM Na molybdate, and 1 mM EDTA with protease inhibitor; Sigma-Aldrich) on ice for 15 minutes. VEGF protein levels in the supernatant was considered to total protein (Bio-Rad, Hercules, CA). To detect the protein levels of CCL2/MCP-1 (R&D Systems), which is a potent chemotactic for monocytes/macrophages, and ICAM-1 (R&D Systems) in the RPE/choroid, 2 days after injury with 25 laser spots, the eyes were enucleated and the RPE/choroid isolated by the same procedure using VEGF ELISA. Duplicate measurements were performed in a masked fashion by an operator not involved in photocoagulation or imaging.

**Flow Cytometry**

Single-cell suspensions isolated from murine RPE/choroid via collagenase D (20 U/mL; Worthington, Lakewood, NJ) were treated in Fc block (purified rat anti-mouse CD16/CD32 monoclonal antibody, 0.5 mg/mL; BD Biosciences, San Jose, CA) for 15 minutes on ice, and stained with Cy5-rat antibody anti-mouse F4/80 (1:30; Serotec, Raleigh, NC). Live cells were detected by gating on forward versus side scatter, followed by analysis of F4/80 in the fluorescence channel (FACS Calibur; BD Biosciences). At least 100,000 viable cells were analyzed per condition and the results were analyzed (Cellquest software; BD Biosciences).

**Statistical Analysis**

The mean areas of CNV were compared by using a linear mixed model with a split-plot, repeated-measures design: The whole plot factor was the genetic group to which the animal belonged, whereas the split plot factor was the eye. \( P = 0.05 \) was considered significant. Results are expressed as the mean ± SEM. A post hoc comparison of means was constructed with Bonferroni adjustment for multiple comparisons (\( P < 0.017 \)). VEGF data and results of flow cytometry were analyzed (Excel; Microsoft, Redmond, WA). \( P < 0.05 \) was considered significant.

**RESULTS**

The volume of CNV 2 weeks after laser injury was smaller in all treatment groups compared with control mice (Fig. 1). The mean CNV volume was significantly smaller in the AS1 (44.16 ± 4.67 × 10⁴ μm³; nonpaired t-test, \( P = 0.0004 \)), AS2 (36.49 ± 4.64 × 10⁴ μm³; \( P < 0.0001 \)), AS10 (25.75 ± 2.41 × 10⁴ μm³; \( P < 0.0001 \)), and AS20 (33.24 ± 8.42 × 10⁴ μm³; \( P < 0.0001 \)) groups compared with control mice (64.21 ± 2.27 × 10⁴ μm³) 2 weeks after laser injury (Fig. 2). However, the volume of CNV in the AS20 group tended to be larger than in the AS10 group (\( P = 0.06 \)).

The mean VEGF level in the RPE/choroid, which peaked on day 3, was significantly reduced in the AS10 group (62.3% ± 4.7%, \( P = 0.001 \)) compared with the controls (Fig. 3).

The mean CCL2/MCP-1 level in the RPE/choroid were not detected in the laser groups. Two days after laser injury in the control group, the CCL2/MCP-1 level had markedly upregulated in the RPE/choroid. The levels decreased in the treatment groups in a dose-dependent manner (Fig. 4), and in the AS10 group, the level was significantly (\( P = 0.02 \)) less than in the control group.

In normal untreated retina, no macrophages were detected in the RPE/choroid. In contrast, after laser injury, macrophages infiltrated the site of the laser injury within 1 day, with a peak response on day 3 coinciding with the peak VEGF expression, followed by rapid resolution by day 5. The number of choroid-infiltrating macrophages decreased markedly in atorvastatin-treated mice compared with control mice (Fig. 5).

There was no significant different in ICAM-1 expression in the RPE/choroid between each treated group and the control group (Fig. 6).

Regarding the VEGF ELISA results, CCL2/MCP-1 ELISA and flow cytometry analyses are shown with no data from the AS20 group, because there were no significant differences in the treated groups in the preliminary experiments and to simplify presentation of the results.
The present study showed that atorvastatin, an HMG-CoA reductase inhibitor, inhibits laser-induced CNV by suppressing macrophage infiltration into the RPE/choroid. These results agree with recent studies that the immune response mediated by macrophages and monocytes plays an important role in CNV growth in an animal model.6 There is growing evidence that leukocyte-mediated angiogenesis involves the interaction of cellular adhesion molecules and VEGF. More recently, Nozaki et al.21 reported that drusen components of complement play important roles in leukocyte recruitment and CNV progression.

In vitro, statins modulate the adhesion cascade at multiple points by targeting the endothelium and leukocytes. Statins affect cellular adhesion by inhibiting chemokine expression in activated leukocytes and endothelial cells. They also decrease ICAM-1 expression in stimulated endothelial cells and monocytes.22–25 Some statins (e.g., lovastatin, simvastatin) bind to the ICAM-1 binding domain of lymphocyte function-associated antigen (LFA)-1, reducing the interaction mediated by ICAM-1/LFA-1. However, in our animal model, there was no significant difference in ICAM-1 expression in the RPE/choroid between the groups treated with the statin and the control group.

In a clinical report,26 low-dose atorvastatin significantly decreased serum MCP-1. Lovastatin and simvastatin inhibited MCP-1 production in a dose-dependent manner in vitro and in vivo.27 Monocyte recruitment is an early step in the initiation of inflammatory and angiogenic processes, and MCP-1 plays an important role in monocyte recruitment. Similar to that report,
our results showed that the mean CCL2/MCP-1 protein levels decreased significantly in the treatment groups.

Recently, statin therapy has been reported to be associated with decreased rates of CNV among patients with AMD.\textsuperscript{15} However, the relationship between statin use and AMD is unclear. In contrast to complex clinical research, animal models allow discrimination of the potential direct anti-inflammatory activity of statins from the lipid-lowering activity.\textsuperscript{28}

Diomede et al.\textsuperscript{28} reported that lovastatin inhibited leukocyte recruitment in an animal model of acute inflammation at oral doses of 5 to 10 mg/kg\textsuperscript{-1}. The effect on leukocyte migration was associated with downregulation of MCP-1 and RANTES (regulated on activation, normal T-cell expressed and secreted) and the cytokine interleukin-6.

Consistent with these results, Kimura et al.\textsuperscript{29} showed that fluvastatin treatment (6 mg/kg\textsuperscript{-1}) of hypercholesterolemic rats reduced the number of leukocytes that adhered to postcapillary venules in response to platelet-activating factor or leukotriene B4. Stalker et al.\textsuperscript{30} found that systemic rosuvastatin (0.5–1.25 mg/kg\textsuperscript{-1}) attenuated thrombin-induced leukocyte rolling, adhesion, and transmigration in normocholesterolemic rats.

Our results suggest that atorvastatin (10 mg/kg) inhibits laser-induced leukocyte recruitment to the RPE/choroid. However, given the limitations of animal models for studying human disease and the relatively high drug concentrations used, the contribution of the anti-inflammatory and antiadhesive effects of statins observed in animal models to their overall benefit in patients remains uncertain.

Macrophages produce VEGF in response to laser injury.\textsuperscript{6} In the current results, atorvastatin decreased VEGF expression in the RPE/choroid induced by laser injury, suggesting that the mechanism of CNV inhibition was suppression of macrophage recruitment through decreased CCL2/MCP-1 expression.

Statins prevent activation of monocytes into macrophages and inhibit production of proinflammatory cytokines, C-reactive protein, and cellular adhesion molecules. They also decrease adhesion of monocytes to endothelial cells, reduce MCP-1 production and macrophage recruitment,\textsuperscript{25} and reduce VEGF expression in coronary disease.\textsuperscript{31} Accordingly, statins exert cardiovascular benefits through direct antiatherogenic properties in the arterial wall beyond their effects on plasma lipids.

Zambarakji et al.\textsuperscript{32} reported that low-dose (0.18 mg/kg per day) pitavastatin reduced fluorescein leakage of laser-induced CNV, but a higher dose (18 mg/kg per day) increased fluorescein leakage and there was a trend toward an increase in CNV size. Likewise, in our results, it was noteworthy that the mean CNV volume in the AS20 group was larger than in the AS10 group, a difference that reached significance. This result sug-
gests that a toxic effect may induce angiogenesis based on the dose of atorvastatin.

Current treatment options are limited to the late stage of the disease when the central vision is already greatly threatened, and even new treatments have little impact on the rate of blindness. Therefore, it is important to define the mechanism of AMD’s progression and prevention. More large clinical trials on statin use and the frequency of AMD are suggested.

References


