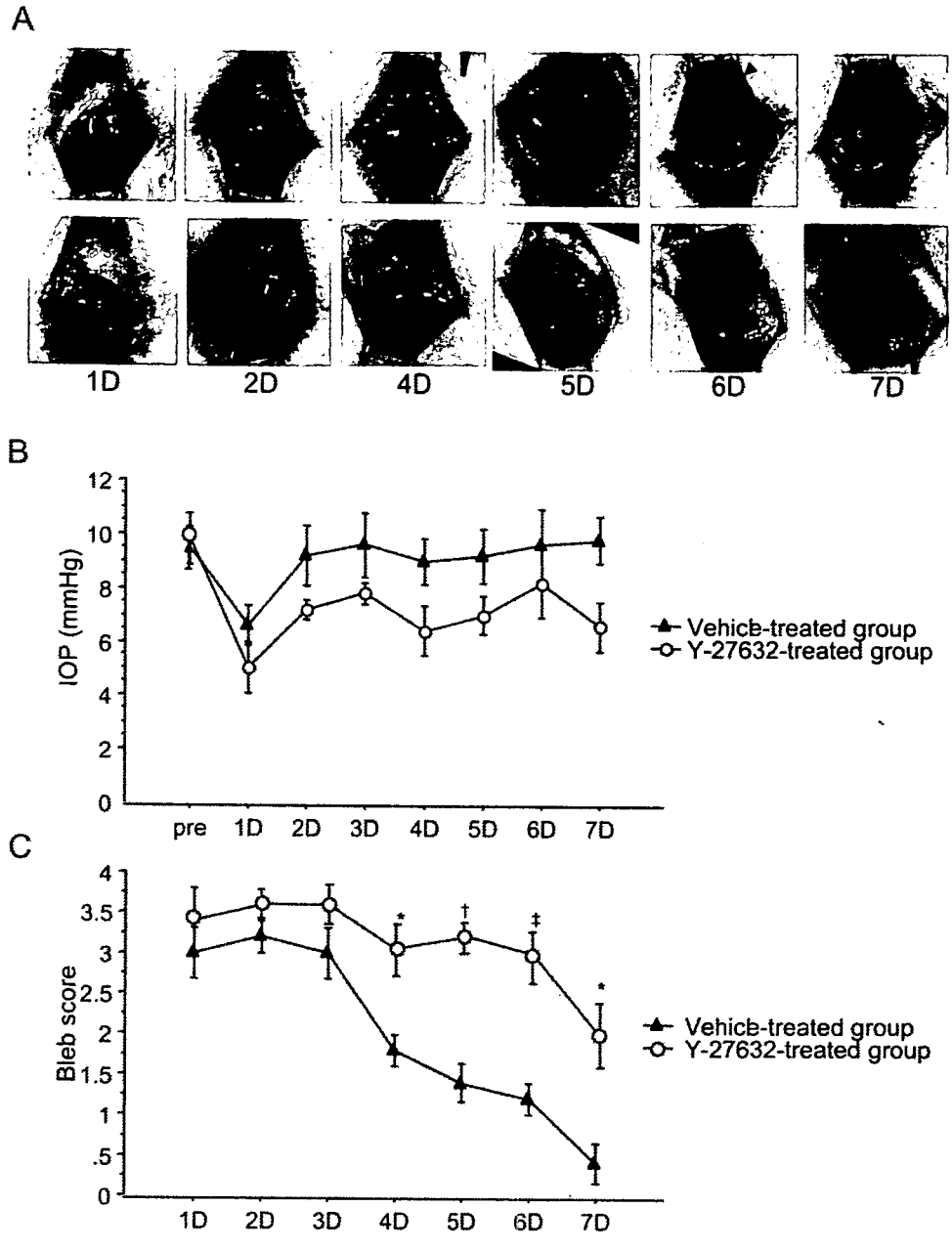

FIGURE 5. Postoperative IOP, bleb features, and bleb scores. (A) Representative postoperative photographs show surviving conjunctival blebs in the Y-27632-treated group that remained diffusely elevated (*top*). A flat, scarred, vascularized bleb, formed after vehicle treatment, is an example of a bleb that did not survive (*bottom*). *Arrows*: edges of the blebs. (B) Postoperative IOP changes in the two groups showed significance between the Y-27632-treated group and the vehicle-treated group 7 days ($P < 0.05$) after surgery. (C) Bleb scores for rabbit eyes treated with Y-27632 or vehicle. The mean ± SEM for six eyes for each treatment is shown for 7 postoperative days. Y-27632-treated eyes exhibited significantly higher bleb scores compared with the control during the 7 days ($P < 0.001$). Y-27632-treated eyes exhibited significantly higher bleb scores compared with the control from days 4 to 7. (*$P < 0.05$, †$P < 0.001$, ‡$P < 0.005$ versus control.)

Gel contraction experiments using three-dimensional cultures of HTFs embedded in collagen type I gels revealed that addition of Y-27632 decreased contraction of gels. This result suggests reduced cell contractility and/or altered interaction between HTFs and collagen type I. Y-27632 treatment increased cell adhesion to collagen type I, and so the change in gel contraction is thought to be due to the relaxation of the HTFs induced by Y-27632.

Our results indicate that administration of Y-27632 enhanced the motility of the HTFs, which is in agreement with the idea that stabilization of actin stress fibers limits cell movement. We have reported that Y-27632 enhances motility of cultured TM cells.[19] In the same study, we demonstrated reduced levels of phosphorylated LIM kinase 2 and cofilin, which is one of the targets downstream of Rho signaling pathways. A previous report from other laboratories showed that constitutively active RhoAV14 significantly inhibited wound closure and that incubation with Y-27632 promotes wound healing.[20] As shown by others[49,50] and according to findings in the present study, inhibition of formation of stress fibers and focal adhesions via Y-27632 improves cell migration during wound healing. Several candidate antiscarring agents, such as alkylphosphocholines and p38 inhibitors, are also reported to inhibit HTF proliferation and contraction.[3,4] Although alkylphosphocholines are reported to inhibit migration of HTFs,[3] the main effect of p38 inhibitors is supposed to be an inhibition of transdifferentiation of HTFs into myofibroblasts.[4] In contrast, it is interesting that we found that Y-27632 not only increased migration but also inhibited cell contraction and transdifferentiation of HTFs into myofibroblasts. These findings, taken together, suggest that inhibition of migration may not be necessary for antiscarring effects when associated with decreased myofibroblast transdifferentiation and inhibited cell contraction. However, further studies are needed to elucidate the association of migration and scar formation after Y-27632 treatment.

In an animal model of filtration surgery, obstruction of the sclerostomy site from excessive ECM deposition and contrac-

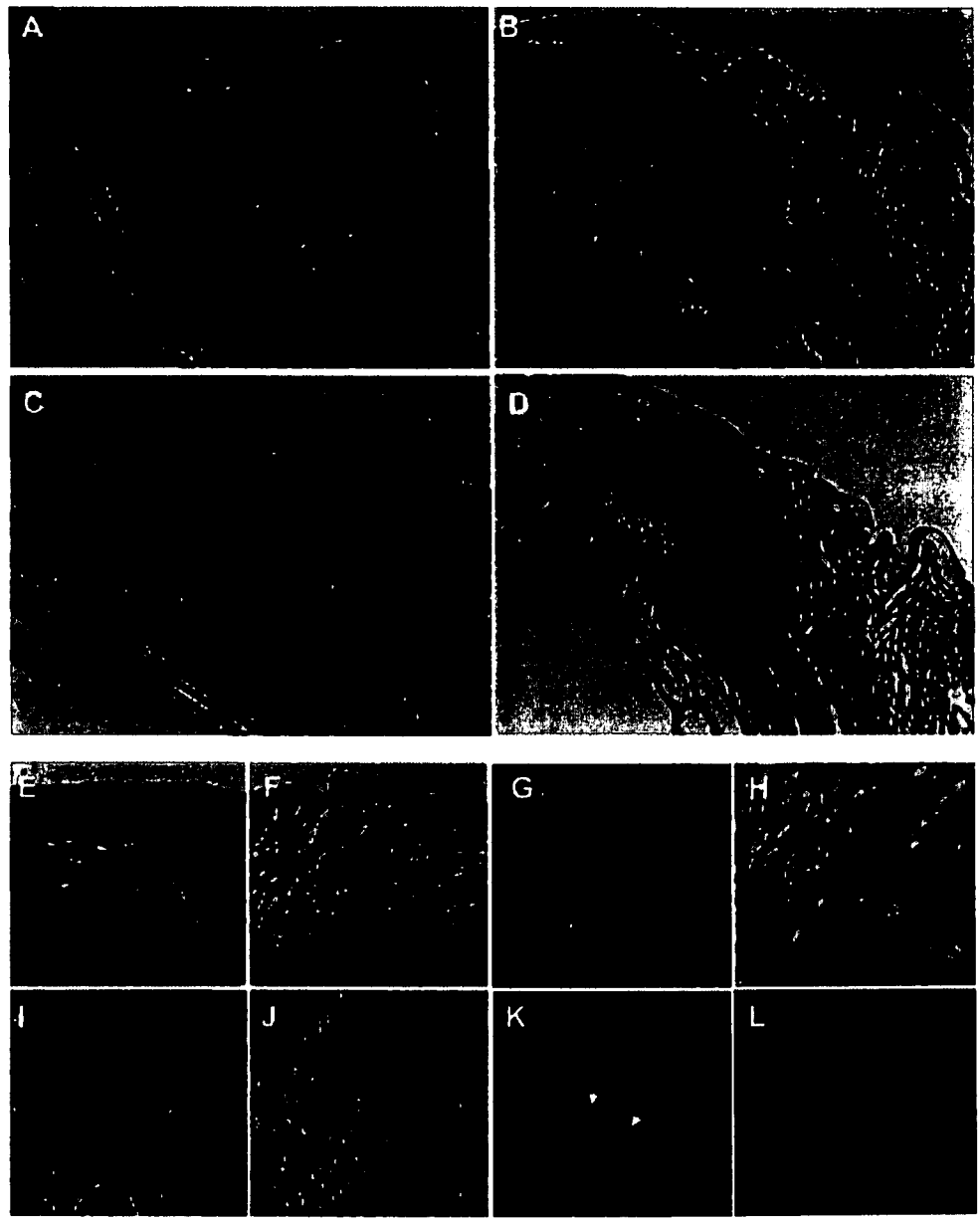

FIGURE 6. Histologic characteristics of postoperative blebs. Y-27632 treatment reduced scarring at the microscopic level. The images show representative sections from eyes in each group. HE stain revealed that the total amount of scar tissue in the subconjunctival space was significantly greater in vehicle-treated (A) than in Y-27632-treated (B) eyes at day 7. EVG stain revealed that vehicle-treated eyes contained scar tissue with densely packed collagen deposits (C, *arrows*) and that the size of the collagen deposits was significantly greater than that in Y-27632-treated eyes (D, *arrows*). EVG also showed that subconjunctival scarring in a vehicle-treated failed bleb consisted of dense collagen fibers and fibroblasts (E, *arrows*) and that the failed bleb area was densely packed with collagen deposits and fibroblasts (G). In contrast, the surviving Y-27632-treated bleb showed much looser architecture with a visible conjunctiva (F) and bleb formation with less collagen (H). In the area of sclerostomy, the wound site of the failed vehicle-treated bleb consisted of densely packed collagen (I, *arrows*). The sclerostomy site in the Y-27632-treated group, different from that in the vehicle-treated group, showed loose cell infiltration without significant collagen deposition (J). Immunohistochemical staining for α-SMA in postoperative blebs on day 7 demonstrated reduced α-SMA expression after Y-27632 treatment (L) compared with massive α-SMA expression of the control eye (K, *white arrows*). Magnification: (A–D) ×25; (E, F, K, L) ×100; (G–J) ×200.

tion due to transdifferentiation of fibroblasts into myofibroblasts, which is characterized by synthesis of α-SMA, have been reported.[51,52] In our present study, histologic analysis of rabbit tissues showed that topical instillation of Y-27632 significantly reduced subconjunctival collagen deposition compared with controls. Y-27632 also significantly reduced the population of cells expressing α-SMA, which indicates inhibition of myofibroblast transdifferentiation in vivo. These observations are in good agreement with the present in vitro data that Y-27632 significantly reduced α-SMA expression in HTFs, suggesting that beneficial effects of Y-27632 in glaucoma surgery may be mediated by reduced cell contraction and inhibition of transdifferentiation of fibroblasts.

Previously, we have reported the significant IOP-lowering effects of ROCK inhibitors and have shown the possibilities for clinical use.[18,53] We have already conducted a phase I clinical trial for the ROCK inhibitor as an IOP-lowering agent and have demonstrated its safety and effectiveness.[54] The present study showed that ROCK inhibitors may have beneficial effects, not only for IOP-lowering but also for bleb formation after filtering surgery. However, further studies are needed to determine the optimal dosage for antiscarring effects. In addition, no direct comparisons were made with effects of MMC. Therefore, we are currently performing a comparison of surgical outcomes for use of Y-27632 and MMC.

In conclusion, our results indicate that Y-27632, a selective ROCK inhibitor, effectively reduced subconjunctival scarring after experimental glaucoma filtration surgery. Y-27632 was safe and well tolerated in this model. Further study is needed to determine whether inhibition of the ROCK/ROK family can lead to development of an alternative, more physiological agent to protect against postoperative scarring.

References

1. Yoon PS, Singh K. Update on antifibrotic use in glaucoma surgery, including use in trabeculectomy and glaucoma drainage implants and combined cataract and glaucoma surgery. *Curr Opin Ophthalmol.* 2004;15:141–146.
2. Mead AL, Wong TT, Cordeiro MF, Anderson IK, Khaw PT. Evaluation of anti-TGF-β2 antibody as a new postoperative anti-scarring agent in glaucoma surgery. *Invest Ophthalmol Vis Sci.* 2003;44:3394–3401.

3. Eibl KH, Banas B, Kook D, et al. Alkylphosphocholines: a new therapeutic option in glaucoma filtration surgery. *Invest Ophthalmol Vis Sci.* 2004;45:2619-2624.
4. Meyer-ter-Vehn T, Gebhardt S, Sebald W, et al. p38 inhibitors prevent TGF-β-induced myofibroblast transdifferentiation in human tenon fibroblasts. *Invest Ophthalmol Vis Sci.* 2006;47:1500-1509.
5. Ehrlich HP, Rajaratnam JB. Cell locomotion forces versus cell contraction forces for collagen lattice contraction: an in vitro model of wound contraction. *Tissue Cell.* 1990;22:407-417.
6. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol.* 2002;3:349-363.
7. Hinz B, Mastrangelo D, Iselin CE, Chaponnier C, Gabbiani G. Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. *Am J Pathol.* 2001;159:1009-1020.
8. Desmouliere A, Chaponnier C, Gabbiani G. Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen.* 2005;13:7-12.
9. Morishige K, Shimokawa H, Eto Y, et al. Adenovirus-mediated transfer of dominant-negative rho-kinase induces a regression of coronary arteriosclerosis in pigs in vivo. *Arterioscler Thromb Vasc Biol.* 2001;21:548-554.
10. Kaibuchi K, Kuroda S, Amano M. Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu Rev Biochem.* 1999;68:459-486.
11. Somlyo AP, Somlyo AV. Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol.* 2000;522:177-185.
12. Fukata Y, Amano M, Kaibuchi K. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol Sci.* 2001;22:32-39.
13. Hall A. Rho GTPases and the actin cytoskeleton. *Science.* 1998; 279:509-514.
14. Ridley AJ. Rho GTPases and cell migration. *J Cell Sci.* 2001;114: 2713-2722.
15. Ishizaki T, Maekawa M, Fujisawa K, et al. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J.* 1996; 15:1885-1893.
16. Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K, Narumiya S. ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett.* 1996; 392:189-193.
17. Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol.* 2003;4:446-456.
18. Honjo M, Tanihara H, Inatani M, et al. Effects of rho-associated protein kinase inhibitor Y-27632 on intraocular pressure and outflow facility. *Invest Ophthalmol Vis Sci.* 2001;42:137-144.
19. Koga T, Awai M, Tsutsui J, Yue BY, Tanihara H. Rho-associated protein kinase inhibitor, Y-27632, induces alterations in adhesion, contraction and motility in cultured human trabecular meshwork cells. *Exp Eye Res.* 2006;82:362-370.
20. Nobes CD, Hall A. Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J Cell Biol.* 1999;144:1235-1244.
21. Meyer-ter-Vehn T, Sieprath S, Katzenberger B, Gebhardt S, Grehn F, Schlunck G. Contractility as a prerequisite for TGF-β-induced myofibroblast transdifferentiation in human tenon fibroblasts. *Invest Ophthalmol Vis Sci.* 2006;47:4895-4904.
22. Jain PT, Pento JT, Graves DC. Cell-growth quantitation methods for the evaluation of antiestrogens in human breast cancer cells in culture. *J Pharmacol Toxicol Methods.* 1992;27:203-207.
23. Zhou L, Zhang SR, Yue BY. Adhesion of human trabecular meshwork cells to extracellular matrix proteins: roles and distribution of integrin receptors. *Invest Ophthalmol Vis Sci.* 1996;37:104-113.
24. Nakamura Y, Hirano S, Suzuki K, Seki K, Sagara T, Nishida T. Signaling mechanism of TGF-β1-induced collagen contraction mediated by bovine trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 2002;43:3465-3472.
25. Nakamura Y, Sagara T, Seki K, Hirano S, Nishida T. Permissive effect of fibronectin on collagen gel contraction mediated by bovine trabecular meshwork cells. *Invest Ophthalmol Vis Sci.* 2003;44:4331-4336.
26. Yamanaka O, Saika S, Okada Y, Ooshima A, Ohnishi Y. Effects of interferon-γ on human subconjunctival fibroblasts in the presence of TGFβ1: reversal of TGFβ-stimulated collagen production. *Graefes Arch Clin Exp Ophthalmol.* 2003;241:116-124.
27. Perkins TW, Faha B, Ni M, et al. Adenovirus-mediated gene therapy using human p21WAF-1/Cip-1 to prevent wound healing in a rabbit model of glaucoma filtration surgery. *Arch Ophthalmol.* 2002;120:941-949.
28. Migdal C, Gregory W, Hitchings R. Long-term functional outcome after early surgery compared with laser and medicine in open-angle glaucoma. *Ophthalmology.* 1994:101:1651-1656; discussion 1657.
29. Khaw PT, Occleston NL, Schultz G, Grierson I, Sherwood MB, Larkin G. Activation and suppression of fibroblast function. *Eye.* 1994;8:188-195.
30. Occleston NL, Daniels JT, Tarnuzzer RW, et al. Single exposures to antiproliferatives: long-term effects on ocular fibroblast wound-healing behavior. *Invest Ophthalmol Vis Sci.* 1997;38: 1998-2007.
31. Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-b1 induces α-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol.* 1993;122:103-111.
32. Grotendorst GR. Connective tissue growth factor: a mediator of TGF-β action on fibroblasts. *Cytokine Growth Factor Rev.* 1997; 8:171-179.
33. Leask A, Abraham DJ. TGF-β signaling and the fibrotic response. *FASEB J.* 2004;18:816-827.
34. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor-b1 induces prolonged severe fibrosis in rat lung. *J Clin Invest.* 1997; 100:768-776.
35. Gabbiani G, Ryan GB, Majne G. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia.* 1971;27:549-550.
36. Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G. A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol.* 1986; 103:2787-2796.
37. Khaw PT, Ward S, Porter A, Grierson I, Hitchings RA, Rice NS. The long-term effects of 5-fluorouracil and sodium butyrate on human Tenon's fibroblasts. *Invest Ophthalmol Vis Sci.* 1992;33:2043-2052.
38. Khaw PT, Doyle JW, Sherwood MB, Smith MF, McGorray S. Effects of intraoperative 5-fluorouracil or mitomycin C on glaucoma filtration surgery in the rabbit. *Ophthalmology.* 1993;100:367-372.
39. Mac I, Soltau JB. Glaucoma-filtering bleb infections. *Curr Opin Ophthalmol.* 2003;14:91-94.
40. Anand N, Arora S, Clowes M. Mitomycin C augmented glaucoma surgery: evolution of filtering bleb avascularity, transconjunctival oozing, and leaks. *Br J Ophthalmol.* 2006;90:175-180.
41. Worthylake RA, Burridge K. RhoA and ROCK promote migration by limiting membrane protrusions. *J Biol Chem.* 2003;278:13578-13584.
42. Hla T, Lee MJ, Ancellin N, Paik JH, Kluk MJ. Lysophospholipids: receptor revelations. *Science.* 2001;294:1875-1878.
43. Yin Z, Watsky MA. Chloride channel activity in human lung fibroblasts and myofibroblasts. *Am J Physiol.* 2005;288:L1110-L1116.
44. Watsky MA. Lysophosphatidic acid, serum, and hyposmolarity activate Cl- currents in corneal keratocytes. *Am J Physiol.* 1995; 269:C1385-C1393.
45. Wong TT, Mead AL, Khaw PT. Matrix metalloproteinase inhibition modulates postoperative scarring after experimental glaucoma filtration surgery. *Invest Ophthalmol Vis Sci.* 2003;44:1097-1103.
46. Tomasek JJ, Vaughan MB, Kropp BP, et al. Contraction of myofibroblasts in granulation tissue is dependent on Rho/Rho kinase/myosin light chain phosphatase activity. *Wound Repair Regen.* 2006;14:313-320.
47. Parizi M, Howard EW, Tomasek JJ. Regulation of LPA-promoted myofibroblast contraction: role of Rho, myosin light chain kinase, and myosin light chain phosphatase. *Exp Cell Res.* 2000;254:210-220.

48. Tangkijvanich P, Melton AC, Santiskulvong C, Yee HF Jr. Rho and p38 MAP kinase signaling pathways mediate LPA-stimulated hepatic myofibroblast migration. *J Biomed Sci.* 2003;10:352–358.
49. Narumiya S, Ishizaki T, Watanabe N. Rho effectors and reorganization of actin cytoskeleton. *FEBS Lett.* 1997;410:68–72.
50. Amano M, Fukata Y, Kaibuchi K. Regulation and functions of Rho-associated kinase. *Exp Cell Res.* 2000;261:44–51.
51. Darby IA, Hewitson TD. Fibroblast differentiation in wound healing and fibrosis. *Int Rev Cytol.* 2007;257:143–179.
52. Miller MH, Grierson I, Unger WI, Hitchings RA. Wound healing in an animal model of glaucoma fistulizing surgery in the rabbit. *Ophthalmic Surg.* 1989;20:350–357.
53. Tokushige H, Inatani M, Nemoto S, et al. Effects of topical administration of y-39983, a selective Rho-associated protein kinase inhibitor, on ocular tissues in rabbits and monkeys. *Invest Ophthalmol Vis Sci.* 2007;48:3216–3222.
54. Tanihara H, Inatani M, Honjo M, et al. Intraocular pressure lowering effects and safety of topical administration of a selective ROCK inhibitor, SNJ-1656, in normal volunteers. *Arch Ophthalmol.* In press.

Current Eye Research, 32:991–997, 2007

ISSN: 0271-3683 print / 1460-2202 online
DOI: 10.1080/02713680701649603

informa
healthcare

Pitavastatin: Protection against Neuronal Retinal Damage Induced by Ischemia-Reperfusion Injury in Rats

Takahiro Kawaji, Yasuya Inomata, Akiomi Takano, Nina Sagara, Masaru Inatani, Mikiko Fukushima, and Hidenobu Tanihara
Department of Ophthalmology and Visual Science, Graduate School of Medical Sciences, Kumamoto University, Honjo, Kumamoto, Japan

Megumi Honjo
Department of Ophthalmology, Kitano Hospital, Ogimachi, Kita-ku, Osaka, Japan

ABSTRACT *Purpose*: To evaluate the neuroprotective effects of pitavastatin against neuronal retinal damage induced by ischemia-reperfusion injury in rats. *Methods and Results*: Ischemia-reperfusion injury was induced in Sprague-Dawley rats using ocular hypertension. Pitavastatin (0.1, 0.5, or 1.0 mg/kg) was given intravenously 12 hr or 5 min before, or 12 or 24 hr after the induction of ischemia-reperfusion injury. Morphometric and retrograde labeling analyses revealed neuroprotective effects when pitavastatin (0.5 mg/kg) was administered 5 min before–even 12 and 24 hr–after induction of ischemia-reperfusion injury. These effects depended on dose; protection was noted at pitavastatin concentrations of 0.5 and 1 mg/kg but not 0.1 mg/kg. Furthermore, preadministration of pitavastatin (0.5 mg/kg) reduced expression of P-selectin and intercellular adhesion molecule-1 at 12 and 24 hr after induction of ischemia-reperfusion injury. *Conclusions*: As pitavastatin was efficacious in preventing retinal neuronal death, it may be a novel therapeutic modality for ischemic retinal diseases.

KEYWORDS ischemia-reperfusion injury; ischemic retinal disease; neuroprotection; pitavastatin

INTRODUCTION

Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, have gained wide acceptance as important agents for treatment of hypercholesterolemia. These inhibitors exert biological effects by blocking conversion of HMG-CoA to mevalonate. Clinical studies of statins have also reported a lower incidence of cerebrovascular and cardiovascular disease independent of the ability of statins to reduce low-density lipoprotein cholesterol levels.[1–5]

The association between the use of statins and vision-threatening diseases, such as age-related macular degeneration (AMD) and diabetic retinopathy, has been evaluated in many clinical and experimental studies; however, the results have been contradictory.[6–14] We previously reported that improvement of endothelial function via the pleiotropic actions of two statins, pravastatin and cerivastatin, could aid in the protection of retinal neurons against damage resulting from ischemic retina.[11] Simvastatin was also reportedly beneficial in inhibition of leukocyte accumulation and vascular permeability in the retina

Received 13 June 2007
Accepted 24 August 2007

Correspondence: Takahiro Kawaji, MD, Ph.D., Department of Ophthalmology and Visual Science, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan. E-mail: kawag@white.plala.or.jp

in a rat model of diabetes.[14] These data suggest the potential of statins to be important drugs for treatment of ischemic retinal diseases.

We report here the neuroprotective effects of pitavastatin (so-called vascular statin), which has a high affinity for vascular endothelium against neuronal retinal damage induced by ischemia-reperfusion injury in rats.

MATERIALS AND METHODS

Animals and Model of Ischemia

Male Sprague-Dawley rats (200–250 g) were used in this study. All experiments were performed in accordance with the Statement for the Use of Animals in Ophthalmic and Visual Research of the Association for Research in Vision and Ophthalmology.

Transient ocular hypertension was induced in the right eye of each rat, according to the method of Rosenbaum et al.,[15] with slight modifications. Rats were anesthetized with a 1:1 mixture of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg). Dilation of the pupil was achieved with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. The anterior chamber of the right eye was cannulated with a 30-gauge needle attached to a line for infusion of balanced salt solution. Intraocular pressure (IOP) was raised to 130 mmHg. Complete non-perfusion was confirmed via an operating microscope. After 60 min of ocular hypertension, the needle was withdrawn and the IOP normalized. The operating microscope was also used to verify reperfusion of the vessels.

Chemicals and Drug Administration

Pitavastatin (trade name: Livalo, code name: NK-104) was generously provided by Kowa (Nagoya, Japan). Carboxymethyl cellulose (CMC) sodium salt, which served as the vehicle for pitavastatin, was obtained from Wako Pure Chemicals (Osaka, Japan). Pitavastatin (0.1, 0.5, or 1.0 mg/kg) and CMC (0.5%) were given intravenously 12 hr or 5 min before or 12 or 24 hr after the induced transient retinal ischemia.

Morphometric Analysis

Eight eyes from 8 rats in each pitavastatin-treated, vehicle-treated, and non-operated control groups were obtained to evaluate the severity of retinal damage. Morphometric analysis was performed in a manner similar to that described previously.[16] Briefly, 7 days after the induced transient retinal ischemia, the rats were killed by an intraperitoneal overdose injection of pentobarbital and the eyes were enucleated. The eyes were immersed in a fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hr at 4°C, followed by dehydration and embedding in paraffin. Transverse sections 4 μm thick were made through the optic disc, were stained with hematoxylin and eosin, and were subjected to morphometric analysis. The degree of hypertension-induced neuronal damage in the retina was quantified by means of cell counts in the ganglion cell layer (GCL) and by measuring the thickness of the inner plexiform layer (IPL) and inner nuclear layer (INL), at 1.5 mm from the optic disc. Data from three sections were averaged for each eye.

Retrograde Labeling of Retinal Ganglion Cells

One day before induction of ischemia, retrograde labeling of GCL cells was performed in a manner similar to that described previously.[17] Briefly, rats were anesthetized with a 1:1 mixture of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg), and then the heads were fixed in a stereotaxic apparatus. Fluoro-Gold (Fluorochrome, Englewood, CO, USA) was microinjected bilaterally into the superior colliculus. Eight days after this injection (i.e., 7 days after the induction of ischemia), the animals were killed by an intraperitoneal overdose injection of pentobarbital and the eyes were enucleated. Eyes were fixed in 4% paraformaldehyde for 1 h. Retinas were divided into six by means of radial cuts, removed from the sclera, and mounted on slides. Fluoro-Gold GCL labeling was analyzed in a manner similar to that described previously.[17] Briefly, regions used for counting the number of GCL cells were selected from two fields in the central area (1 mm from the optic disc) from each of the six radial cuts. Thus, for each eye, 12 fields were evaluated to obtain the labeled GCL counts.

Analysis of Expression of Genes for P-Selectin and Intercellular Adhesion Molecule -1 (ICAM-1) By Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction

Seven eyes from 7 rats in each pitavastatin-treated, vehicle-treated, and non-operated control groups were

used to analyze expression of the adhesion molecules P-selectin and ICAM-1. At 12 or 24 hr after reperfusion, the eyes were enucleated and retinas were collected from posterior segments; control eyes were handled in the same fashion. Total RNA was prepared from these fresh tissue samples by using the AquaPure RNA isolation Kit (BIO-RAD, Hercules, CA, USA), according to the manufacturer's instructions. Real-time one-step reverse transcription-polymerase chain reaction (RT-PCR) was performed with SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA, USA) by using the ABI prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. For RT-PCR analysis, 1 μg of template RNA, 0.2 μM sense primers, 0.2 μM antisense primers, 1 μL of RT/Platinum Taq Mix, and 25 μL of 2× reaction mixture, in a total volume of 50 μL, were used. RNA was reverse-transcribed into cDNA by one cycle at 50°C for 30 min followed by one cycle at 94°C for 2 min. The cDNA was amplified for 40 cycles: 94°C for 15 s, 50°C for 30 s, and 72°C for 1 min (the last cycle at 72°C for 5 min). We used the primers for P-selectin, ICAM-1, and GAPDH designed by TaqMan Gene Expression Assays. Data for quantification were analyzed with the ABI PRISM 7000 analysis software. P-selectin and ICAM-1 mRNA levels were estimated as the ratio of these mRNA copies to GAPDH mRNA copies.

Statistical Analysis

All values were presented as means ± SEM. Data were analyzed via one-way analysis of variance using the post-hoc test with Fisher's protected least significant difference procedure. Differences were considered statistically significant when p values were less than 0.05.

RESULTS

Morphometric Analysis

To investigate the protective effects of pitavastatin against retinal ischemia induced by 60 min of ocular hypertension (130 mm Hg), we performed a quantitative morphometric analysis (Fig. 1). Induction of transient retinal ischemia caused severe destruction of the inner retinal elements, which led to decreased thickness of different layers and damage to retinal cells. In vehicle-treated eyes, the IPL and INL were 17.7 ± 1.1 μm and 22.8 ± 0.6 μm thick, respectively; these values were significantly lower compared with those of the control non-operated eyes, 42.3 ± 1.4 μm and 31.3 ± 1.5 μm, respectively ($p < 0.05$). In addition, the cell density of the GCL in the vehicle-treated eyes, 25.2 ± 1.7 cells/mm, was significantly reduced compared with that in the non-operated controls, 58.2 ± 3.3 cells/mm ($p < 0.05$) (Fig. 1B).

With administration of pitavastatin (0.5 mg/kg) at 5 min before the induction of transient retinal ischemia, destruction of inner retinal elements was significantly suppressed compared with control eyes (Fig. 1A). With pitavastatin treatment, the IPL and INL were 23.7 ± 2.4 μm and 25.7 ± 1.2 μm thick, respectively; both values were significantly higher than those for vehicle-treated eyes ($p < 0.05$) (Fig. 1B). Also, the cell density of the GCL in pitavastatin-treated eyes was 38.9 ± 4.5 cells/mm, which is also significantly greater than that for vehicle-treated eyes ($p < 0.05$).

Analysis of Retrograde Labeling of the GCL

To investigate whether pitavastatin protects the GCL from ischemia-reperfusion induced neuronal death, we used retrograde labeling of GCL with Fluoro-Gold, which allows the individual cells of the GCL to be observed in whole-mount retinas (Fig. 2A). The mean cell density of the GCL was 3882 ± 350 cells/mm^2 and 1725 ± 220 cells/mm^2 in controls and in vehicle-treated eyes, respectively. In contrast, the mean cell density of the GCL in pitavastatin-treated eyes was 2654 ± 302 cells/mm^2, which is significantly higher than that for the vehicle-treated eyes ($p < 0.05$) (Fig. 2B).

Dependence on Dose and Time of the Neuroprotective Effects of Pitavastatin

To elucidate possible dose-dependent relationships, different concentrations of pitavastatin (0.1, 0.5, and 1.0 mg/kg) were administered 5 min before induction of transient retinal ischemia. Our morphometric analysis showed significant differences between pitavastatin- and vehicle-treated eyes at the higher pitavastatin concentrations (Fig. 3).

To evaluate the influence of the timing of administration, pitavastatin was given at 12 hr before, at 5 min before, or at 12 or 24 hr after the induction of

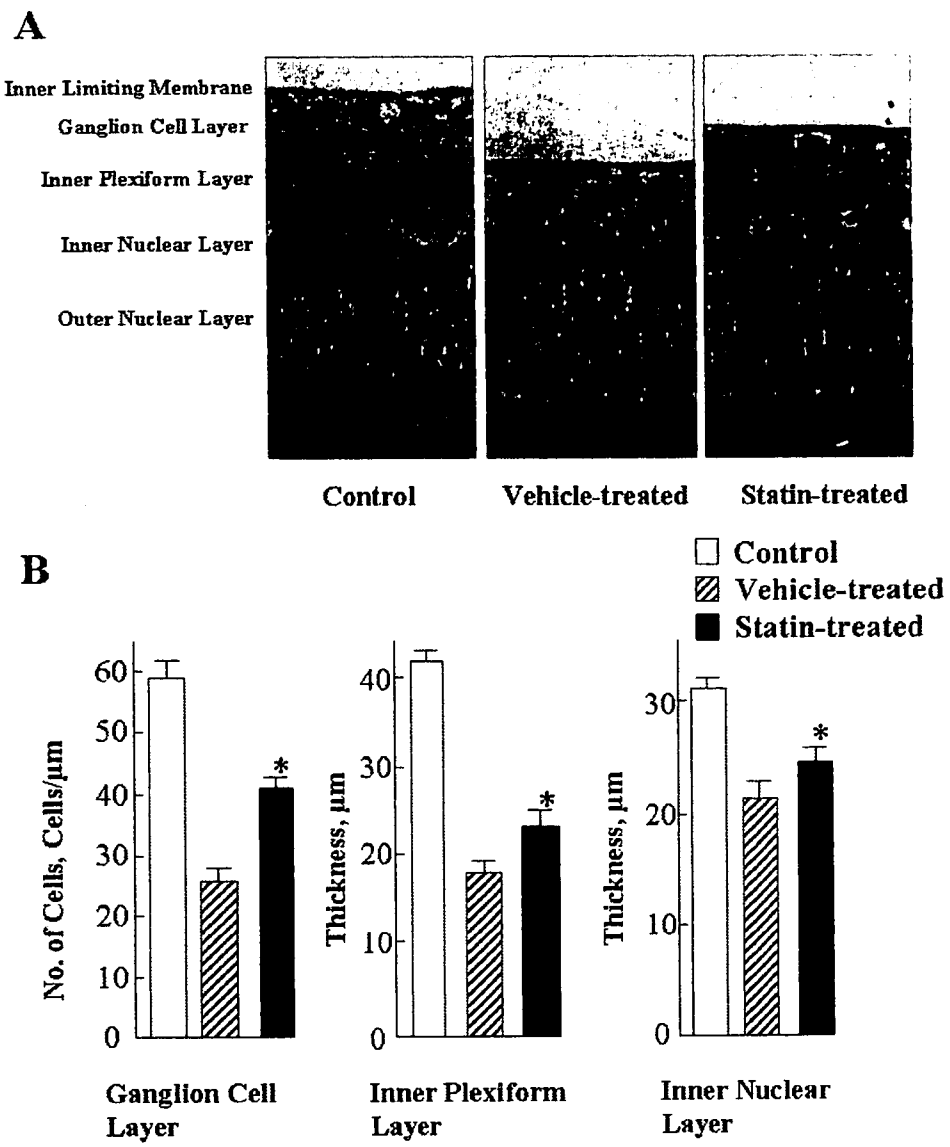

FIGURE 1 Light micrographs of the retina at a distance of 1.5 mm from the center of the optic nerve head. (A) Representative results for non-operated control, vehicle-treated, and pitavastatin-treated eyes 7 days after reperfusion. (B) Number of cells and thickness of different retinal layers at 7 days after reperfusion. Values are means ± SEM. Asterisks indicate significantly different at $p < 0.05$ compared with vehicle-treated eyes. For each group, $n = 8$.

retinal ischemia. In rats given pitavastatin at 5 min before or at 12 and 24 hr after induction of retinal ischemia, the thicknesses of the IPL and INL were significantly greater than those in vehicle-treated eyes ($p < 0.05$) (Fig. 4). In addition, the mean cell densities in the GCL in pitavastatin-treated eyes were statistically higher than those in control eyes and in vehicle-treated eyes ($p < 0.05$) (Fig. 4).

Expression of P-Selectin and ICAM-1 in the Retina

In an effort to investigate pitavastatin-mediated inhibition of a leukocyte-endothelium interaction, we performed real-time quantitative RT-PCR to determine expression levels of adhesion molecules in pitavastatin-treated or -untreated rat retinas. Figure 5 shows that ICAM-1 and P-selectin gene expression was significantly reduced at the time of 12 or 24 hr after reperfusion, which suggests that pitavastatin, like other statins, suppressed activation of endothelial cells after ischemia-reperfusion.[11,14]

DISCUSSION

The association between the long-term use of statins and ocular vascular disease has been evaluated in many clinical studies; however, the results have been

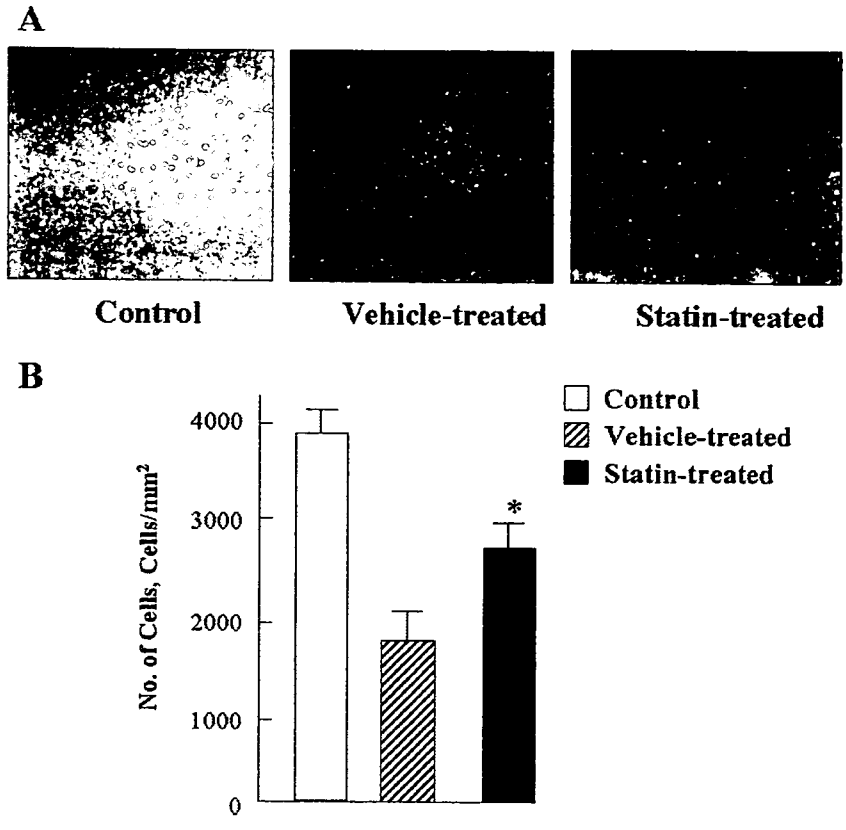

FIGURE 2 Retrograde labeling of the GCL. (A) Fluorescence microscopic photographs of representative retinal sections at 7 days after reperfusion. (B) Number of labeled cells at 7 days after reperfusion. Values are means ± SEM. Asterisks indicate significantly different at $p < 0.05$ compared with vehicle-treated eyes. For each group, $n = 7$.

contradictory.[6–9] The association between ocular diseases and statins may have two possible explanations. First, the cholesterol-lowering effects of statins and the resultant improvement in cardiovascular disease may inhibit the onset and progression of ocular disease, as evidenced by a higher incidence of cardiovascular disease in patients with AMD and diabetic retinopathy than in controls. Another possibility is that the pleiotropic effects, such as improved endothelial function, decreased low-density lipoprotein oxidation, foam cell formation, leukocyte-endothelium interactions, plaque rupture, and smooth muscle cell proliferation[18–20] of statins are therapeutic and impede the progression of retinal diseases. We previously reported that improvement of endothelial function via the pleiotropic actions of two statins, pravastatin and cerivastatin, could protect the retinal neurons against damage resulting from ischemic retina.[11] Furthermore, we recently demonstrated that the therapeutic dose of pitavastatin for human hypocholesterolemia effectively suppressed experimental choroidal neovascularization in rats via the anti-inflammatory and -angiogenic effects of pitavastatin.[13]

Pitavastatin (also known as NK-104) is a recently developed member of the statin family. This agent produces long-lasting (more than 6 hr) inhibition of liver sterol synthesis.[21] Also, its bioavailability in humans is high, with a circulating half-life of just above 13 hr, and it undergoes very slight modification by the cytochrome P450 enzyme system.[22] These characteristics suggested the possibility of long-term safety and stability of this compound in clinical situations. In addition, pitavastatin produces lower blood cholesterol and triglyceride levels at lower doses administered compared with other statins such as atorvastatin.[23,24] In addition to our previous results,[11] these data on pitavastatin encouraged us to investigate this drug as a useful therapy for retinal diseases.

Our data in the present study showed that pitavastatin had neuroprotective effects against transient retinal ischemia similar to those of two other statins, pravastatin and cerivastatin. Furthermore, our morphometric analysis revealed that even administration of pitavastatin at 24 hr after reperfusion produced neuroprotective effects similar to those obtained during the first 12 hr. Thus, administration of this drug may

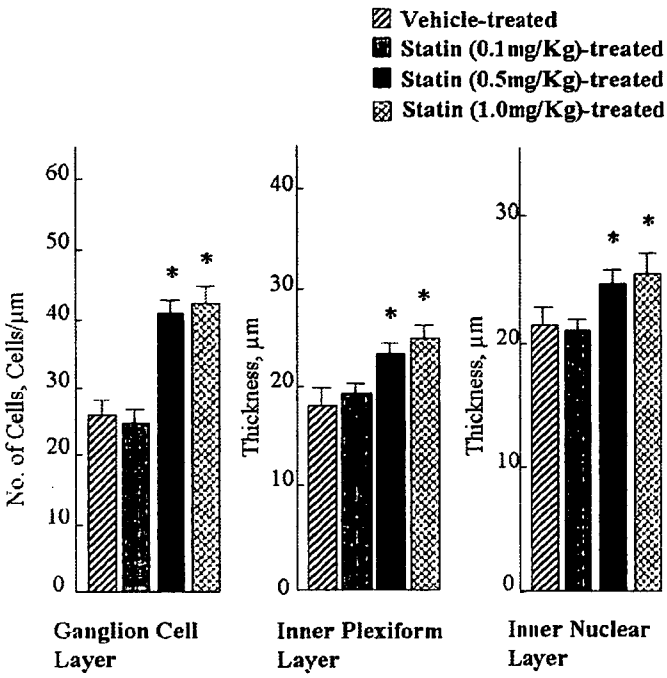

FIGURE 3 Dose dependency of the neuroprotective effects of pitavastatin. Three concentrations of pitavastatin (0.1, 0.5, and 1.0 mg/kg) were administered 5 min before induction of transient retinal ischemia. Representative results are shown for the number of cells in the GCL and the thickness of different retinal layers at 7 days after reperfusion. Values are means ± SEM. Asterisks indicate significantly different at $p < 0.05$ compared with vehicle-treated eyes. For each group, $n = 7$.

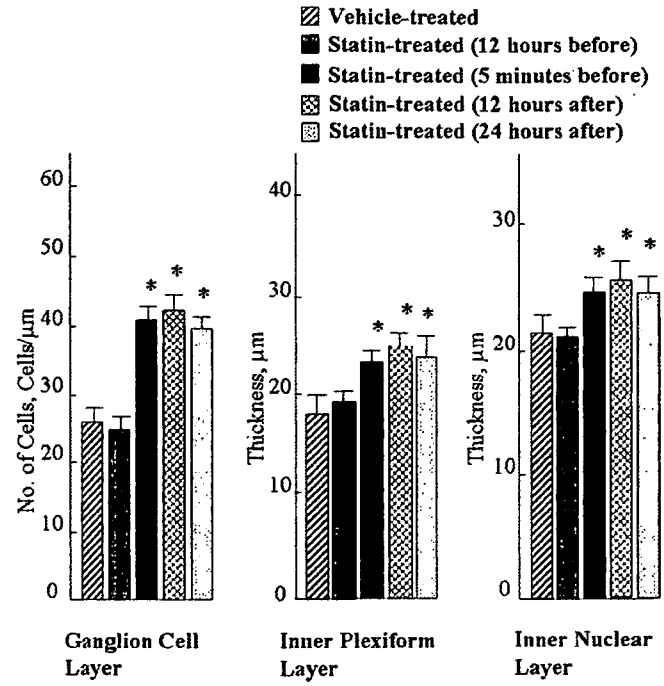

FIGURE 4 Timing of administration of pitavastatin. Pitavastatin (0.5 mg/kg) was given at 12 hr before, 5 min before, or 12 or 24 hr after the induction of transient retinal ischemia. Representative results are shown for the number of cells in the GCL and the thickness of different retinal layers at 7 days after reperfusion. Values are means ± SEM. Asterisks indicate significantly different at $p < 0.05$ compared with vehicle-treated eyes. For each group, $n = 7$.

be effective as an adjunctive treatment even after the onset of ischemic retinal disease, because it would stop progression of the disease in which the improvement of endothelial function is component of the pathogenesis.

Leukocyte adhesion molecules are reported to be upregulated during acute activation of endothelial cells induced by ischemia-reperfusion.[25] We previously clearly demonstrated that the reduction of leukocyte accumulation by inhibition of ICAM-1 and P-selectin markedly diminished the retinal damage from transient retinal ischemia in rats with pravastatin and cerivastatin.[11] In this study, we demonstrated that pitavastatin effectively reduced ICAM-1 and P-selectin expression in the retina, and protected the retina from neuronal death. These findings agree with those of our previous study.[11]

The doses of pitavastatin tested in the current study are higher than that of the normal dose in humans to treat hypercholesterolemia. However, it is generally accepted that the bioequivalent dosage must be higher, because of the higher metabolic rates in rodents. In addition, the medication is given in these animals as a single intravenous dose rather than as a sustained oral administration like human daily use. Despite these differences, our results suggested that therapeutic doses in humans may be sufficient to achieve neuroprotective action. A clinical trial would be necessary to make this determination.

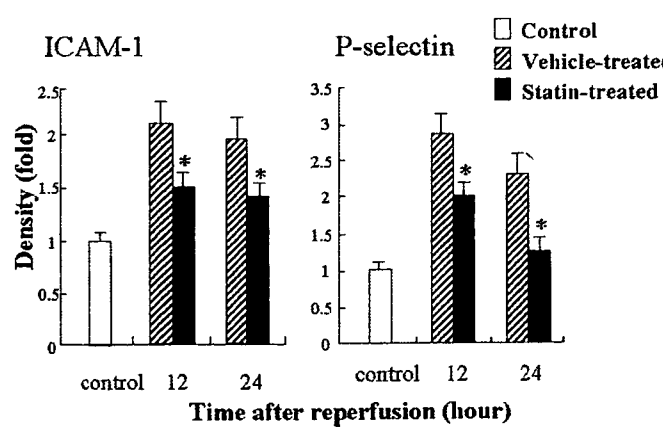

FIGURE 5 ICAM-1 and P-selectin gene expression in the retina after reperfusion. Representative results are given for ICAM-1 and P-selectin gene expression in the rat retina at 12 and 24 hr after reperfusion. ICAM-1 and P-selectin mRNA levels were estimated as the ratio of these mRNA copies to GAPDH mRNA copies. Values are means ± SEM. Asterisks indicate significantly different at $p < 0.05$ compared with vehicle-treated rats. For each group, $n = 7$.

In conclusion, our present study showed the potential of pitavastatin may have application as a novel neuroprotective treatment of ischemic retinal diseases.

ACKNOWLEDGMENTS

The authors' work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, and from the Ministry of Health and Welfare, Japan.

REFERENCES

[1] The Scandinavian Simvastatin Survival Study (4S). .Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease. *Lancet.* 1994;344:1383–1389.

[2] Shepherd J. Statin therapy in clinical practice: new developments. *Curr Opin Lipidol.* 1995;6:254–255.

[3] Treasure CB, Klein JL, Weintraub WS, et al. Beneficial effects of cholesterol-lowering therapy on the coronary endothelium in patients with coronary artery disease. *N Engl J Med.* 1995;332:481–487.

[4] Delanty N, Vaughan CJ. Vascular effects of statins in stroke. *Stroke.* 1997;28:2315–2320.

[5] Sacks FM, Pfeffer MA, Moye LA, et al. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and recurrent events trial investigators. *N Engl J Med.* 1996;335:1001–1009.

[6] Baghdasarian SB, Jneid H, Hoogwerf BJ. Association of dyslipidemia and effects of statins on nonmacrovascular diseases. *Clin Ther.* 2004;26:337–351.

[7] Wilson HL, Schwartz DM, Bhatt HR, et al. Statin and aspirin therapy are associated with decreased rates of choroidal neovascularization among patients with age-related macular degeneration. *Am J Ophthalmol.* 2004;137:615–624.

[8] Klein R, Klein BE, Tomany SC, et al. Relation of statin use to the 5-year incidence and progression of age-related maculopathy. *Arch Ophthalmol.* 2003;121:1151–1155.

[9] McGwin G, Jr., Owsley C, Curcio CA, Crain RJ. The association between statin use and age related maculopathy. *Br J Ophthalmol.* 2003;87:1121–1125.

[10] McGwin G, Jr, McNeal S, Owsley C, et al. Statins and other cholesterol-lowering medications and the presence of glaucoma. *Arch Ophthalmol.* 2004;122:822–826.

[11] Honjo M, Tanihara H, Nishijima K, et al. Statin inhibits leukocyte-endothelial interaction and prevents neuronal death induced by ischemia-reperfusion injury in the rat retina. *Arch Ophthalmol.* 2002;120:1707–1713.

[12] Zambarakji HJ, Nakazawa T, Connolly E, et al. Dose-dependent effect of pitavastatin on VEGF and angiogenesis in a mouse model of choroidal neovascularization. *Invest Ophthalmol Vis Sci.* 2006;47:2623–2631.

[13] Sagara N, Kawaji T, Takano A, et al. Effect of pitavastatin on experimental choroidal neovascularization in rats. *Exp Eye Res.* 2007;84:1074–1080.

[14] Miyahara S, Kiryu J, Yamashiro K, et al. Simvastatin inhibits leukocyte accumulation and vascular permeability in the retinas of rats with streptozotocin-induced diabetes. *Am J Pathol.* 2004;164:1697–1706.

[15] Rosenbaum DM, Rosenbaum PS, Gupta A, et al. Retinal ischemia leads to apoptosis which is ameliorated by aurintricarboxylic acid. *Vision Res.* 1997;37:3445–3451.

[16] Morizane C, Adachi K, Furutani I, et al. N(omega)-nitro-L-arginine methyl ester protects retinal neurons against N-methyl-D-aspartate-induced neurotoxicity in vivo. *Eur J Pharmacol.* 1997;328:45–49.

[17] Sawada A, Neufeld AH. Confirmation of the rat model of chronic, moderately elevated intraocular pressure. *Exp Eye Res.* 1999;69:525–531.

[18] Rosenson RS, Tangney CC. Antiatherothrombotic properties of statins: implications for cardiovascular event reduction. *JAMA.* 1998;279:1643–1650.

[19] Laufs U, Liao JK. Direct vascular effects of HMG-CoA reductase inhibitors. *Trends Cardiovasc Med.* 2000;10:143–148.

[20] Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Arterioscler Thromb Vasc Biol.* 2001;21:1712–1719.

[21] Aoki T, Nishimura H, Nakagawa S, et al. Pharmacological profile of a novel synthetic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Arzneimittelforschung.* 1997;47:904–909.

[22] Kojima J, Fujino H, Abe H, et al. Identification of metabolites of NK-104, an HMG-CoA reductase inhibitor, in rat, rabbit and dog bile. *Biol Pharm Bull.* 1999;22:142–150.

[23] Kajinami K, Koizumi J, Ueda K, et al. Effects of NK-104, a new hydroxymethylglutaryl-coenzyme reductase inhibitor, on low-density lipoprotein cholesterol in heterozygous familial hypercholesterolemia. Hokuriku NK-104 Study Group. *Am J Cardiol.* 2000;85:178–183.

[24] Saito Y, Yamada N, Teramoto T, et al. Clinical efficacy of pitavastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, in patients with hyperlipidemia. Dose-finding study using the double-blind, three-group parallel comparison. *Arzneimittelforschung.* 2002;52:251–255.

[25] Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA.* 1991;88:4651–4655.

Long-Term Relationship between Intraocular Pressure and Visual Field Loss in Primary Open-Angle Glaucoma

Masaru Inatani,[1] Keiichiro Iwao,[1] Toshihiro Inoue,[1] Maiko Awai,[1] Takahito Muto,[1] Takahisa Koga,[1] Minako Ogata-Iwao,[2] Ryuhei Hara,[2] Ryusuke Futa,[2] Hidenobu Tanihara[1]

[1]Department of Ophthalmology and Visual Science, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan, and [2]Department of Ophthalmology, Nippon Telegraph and Telephone Corporation West Kyushu General Hospital.

Corresponding author; Masaru Inatani

Department of Ophthalmology and Visual Science, Kumamoto University Graduate School of Medical Sciences, 1-1-1, Honjo, 860-8556 Kumamoto, Japan

E-mail: inatani@fc.kuh.kumamoto-u.ac.jp

Tel.: +81-96-373-5247

Fax: +81-96-373-5249

Statement of conflict of interest: none

This study was supported, in part, by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and from the Ministry of Health and Welfare of Japan.

Abstract

Purpose: To investigate the dependence upon intraocular pressure of the progression of visual field defects in eyes with primary open-angle glaucoma, in which the mean intraocular pressure was maintained at <21 mmHg.

Methods: This study involved 100 eyes with primary open-angle glaucoma, which were followed up for ≥5 years. The mean intraocular pressure levels were maintained at <21 mmHg during the follow-up period. The relationship between the intraocular pressure and the progression of visual field defects, which was scored using the Advanced Glaucoma Intervention Study criteria, was investigated retrospectively.

Results: Compared with the baseline scores, the visual field defect scores had significantly worsened by the end of the follow-up period (p<0.0001, Wilcoxon paired signed rank test). The change in the visual field defect score (2.5±0.5) in eyes with average intraocular pressure levels of ≥16 mmHg (n=36) was significantly greater (p=0.031, Mann Whitney-U test) than the change (1.3±0.3) in eyes with average intraocular pressure levels of <16 mmHg (n=64). Moreover, intraocular pressures of ≥18 mmHg made a major contribution to the aggravation of visual field defects in eyes with primary open-angle glaucoma.

Conclusions: Eyes with primary open-angle glaucoma and with mean intraocular pressure levels maintained at <21 mmHg underwent intraocular pressure-dependent progression of their visual field defects. Our results suggest that further intraocular pressure-lowering would be beneficial in such cases.

Key words: Advanced Glaucoma Intervention Study; cupping/disc ratio; Humphrey visual field analyzer; normal tension glaucoma

Introduction

Intraocular pressure (IOP) is the critical factor influencing the progression of glaucomatous optic nerve damage. Large-scale multicenter studies, including the Advanced Glaucoma Intervention Study (AGIS), the Collaborative Initial Glaucoma Treatment Study (CIGTS), the Ocular Hypertension Treatment Study (OHTS), and the Early Manifest Glaucoma Trial (EMGT), have shown that IOP reduction is effective in counteracting the progression of glaucomatous visual field loss. Furthermore, these and other clinical studies suggest that a target IOP of 21 mmHg, which corresponds to two standard deviations (SD) above the mean IOP of normal eyes (1, 2), is not sufficient to prevent visual field loss in eyes with primary open-angle glaucoma (POAG); this is because the visual field of most advanced glaucomatous eyes remains stable only when the mean IOP is maintained at <15 mmHg (3, 4) or <18 mmHg (5) for long follow-up periods. This implies that the target IOP for preventing the further progression of glaucomatous optic neuropathy might be <21 mmHg. However, it has also been suggested that the progression of glaucomatous optic neuropathy in POAG eyes is more dependent upon other clinical factors, such as circulatory abnormalities and immune responses. Eid *et al.* (6) found no relationship between IOP reduction and the stability of the visual field in eyes treated medically for long follow-up periods. In the present study, we examined whether the progression of visual field defects in POAG eyes with IOPs that were maintained, on average, at <21 mmHg were dependent upon the IOP levels.

Methods

Patients with POAG whose IOP and visual fields were followed up regularly for ≥5 years were included in this retrospective study. Individuals whose baseline visual field defects were measured between 1981 and 2000 at Kumamoto University Hospital, and at Nippon Telegraph and Telephone Corporation West Kyushu General Hospital, were enrolled in the study. We defined the outcomes of the second visual field tests as the baseline data for visual field loss. If the reliability values (fixation losses, false-positive responses, or false-negative responses) were ≥50%, the data obtained in the third or subsequent test that fitted our criteria served as the baseline. We enrolled the patients who received visual field tests at least once a year. The visual field defects were evaluated using the AGIS scores, ranging from 0 (no defect) to 20 (end-stage defect) as described previously (7). Patients with baseline scores of 1 to 16 were enrolled in the study, whereas those with scores of ≥17 were excluded. Between 1981 and 2003, measurements were made using Humphrey visual field analyzers with a full-threshold strategy set for the central 30-2 threshold test. We analyzed 52 test locations, corresponding to areas within the central 24-2 threshold, which did not include sites above or below the center of the blind-spot.

During the follow-up as well as at the baseline, patients were treated with medicine and surgeries for IOP lowering. The IOP levels were evaluated using a Goldmann Applanation Tonometer on a slit-lamp biomicroscope. Initially, the IOP values measured at visits made over a period of 3 months were averaged, and the 3-month average values were then averaged over 1 year; this value was designated as

the average IOP for the year. All of the average yearly IOPs were then averaged, and this value was designated as the average IOP level.

The exclusion criteria were as follows: eyes with a baseline visual acuity of <0.2 in decimal notation according to the Landolt C chart; eyes with an average IOP level of >21 mmHg over the entire follow-up period; and eyes with significant associated visual acuity changes due to senile cataract or other ocular diseases, or due to intraocular surgery (including cataract surgery). POAG cases in which the IOP levels >21 mmHg were not recorded at any time before or during the follow-up periods including the 24-h IOP hospital examinations, and with or without IOP lowering treatment, were defined as normal tension glaucoma (NTG). POAG cases in which IOP levels >21 mmHg were recorded at least once, including at times other than during the follow-up period, were defined as POAG with high pressure. To categorize the eyes with POAG into subgroups based upon the IOP levels, the number of years during which the highest IOP was ≥18 mmHg was determined for each case. Eyes that were classified as POAG with high pressure were classified as either ≤3 years (group A) or ≥4 years (group B). By contrast, NTG eyes were classified as either 0 years (group C) or ≥1 year (group D). To evaluate the probability of progression of the visual field defects during the follow-up period in each group, we calculated Kaplan–Meier survival curves. We defined a deterioration of ≥4 points according to the AIGS criteria as progression of the visual field defects. It is known that 5% of eyes deteriorate by ≥4 points due to inter-test fluctuations [7]; thus, worsening of the visual field defects by ≥4 points was not classified as progression if the visual field defects were classed as less severe in

subsequent tests.

In addition, we evaluated the enlargement of the cup on the basis of the vertical cupping/disc (C/D) ratios. The vertical C/D ratios calculated from the drawings in the clinical records were expressed as decimal values with intervals of 0.1. We analyzed the C/D ratios at the baseline and at the last examination.

Results

In total, 70 eyes with POAG with high pressure and 30 eyes with NTG met the criteria for inclusion in the study. In 14 of 30 eyes with NTG, the 24-h IOP hospital examinations were performed, showing no IOP levels of >21 mmHg. The mean ± SD values for age, follow-up period, IOP level at baseline, and average IOP level during follow-up are presented in Table 1. During the follow-up period, all the eyes and 27 eyes were treated with anti-glaucomatous drugs and glaucoma surgeries, respectively. The mean ± standard error (SE) visual field defect scores at baseline and at the last examination for the 100 eyes were 6.3±0.4 and 8.0±0.5, respectively. This difference was statistically significant ($p<0.0001$, Wilcoxon paired signed rank test). The distribution of each score is 14 eyes in 1 point, 9 eyes in 2 points, 11 eyes in 3 points, 8 eyes in 4 points, 10 eyes in 5 points, 5 eyes in 6 points, 6 eyes in 7 points, 8 eyes in 8 points, 2 eyes in 9 points, 9 eyes in 10 points, 4 eyes in 11 points, 4 eyes in 12 points, 3 eyes in 13 points, 1 eye in 14 points, 2 eyes in 15 points and 4 eyes in 16 points. The baseline scores for the POAG eyes with high pressure (6.5±0.5) and NTG (5.8±0.7) had increased to 8.1±0.6 and 7.8±0.8, respectively, by the time of the last examination,

thereby demonstrating statistically significant progression ($p<0.0001$, Wilcoxon paired signed rank test).

An analysis of the relationship between the average IOP levels during the follow-up period and the increase in visual field defect scores (Fig. 1) showed that high average IOP levels and increased scores were weakly but significantly correlated ($r=0.21$, $p=0.037$, Pearson's correlation coefficient test). In addition, we divided POAG eyes into two groups; high IOP group (average IOP levels of ≥16 mmHg; $n=36$) and low IOP group (average IOP levels of <16 mmHg; $n=64$). The comparison of two groups showed that the increases in the visual field scores of high IOP group (2.5±0.5) were significantly greater ($p=0.031$, Mann Whitney-U test). More specifically, in the high pressure group, the increases in the visual field scores for eyes with IOP levels ≥16 mmHg (2.4±0.5; $n=34$) were greater ($p=0.012$, Mann Whitney-U test) than those for eyes with IOP levels <16 mmHg (0.9±0.4; $n=36$). We further divided the NTG group into two subgroups (≥13.8 mmHg or <13.8 mmHg) based on the median values of the average IOP levels, because only two eyes had average IOP levels ≥16 mmHg. However, no significant difference was found between the increases in scores in these two subgroups: 1.3±0.6 ($n=15$) for the <13.8 mmHg group versus 2.8±0.7 ($n=15$) for ≥13.8 mmHg group ($p=0.073$, Mann-Whitney U test; Fig. 2).

In addition, a comparison of the visual field score changes in groups A and B, and groups C and D (Fig. 3), revealed that the increases in groups B (2.4±0.5; $n=32$) and D (3.2±0.7; $n=13$) were significantly greater ($p=0.014$, Mann-Whitney U test) than those in groups A (0.9±0.3; $n=38$; $p=0.013$, Mann-Whitney U test) and C (1.1±0.5;

n=17; p=0.014, Mann-Whitney U test). A Kaplan–Meier analyses of the relationship between IOP levels and progression of visual field defects (Fig. 4) indicated that the 5-year survival ratios were 89.5% in group A and 84.4% in group B, and the 10-year survival ratios were 83.5% in group A and 44.5% in group B, indicating a poorer prognosis for group B (p=0.047, log rank test). By contrast, although the same comparisons of groups C and D showed values of 76.9% and 76.5%, respectively, at 5 years, and of 0% and 76.5%, respectively, at 10 years, the difference was not significant (p=0.466, log rank test).

Also, to examine correlation between IOP fluctuation and the progression of visual filed defect, we analyzed the relationship between AGIS score change and the mean value of yearly IOP ranges in all the POAG eyes. But, no statistical significance was shown (p=0.80, Pearson's correlation coefficient test).

At baseline, the vertical C/D ratios of 0.8 or less were shown in 56 of 100 eyes. Of 56 eyes with the C/D ratio of $\leq$ 0.8, the C/D ratio increased by $\geq$0.2 during the follow-up period in 18 eyes, and increased by <0.2 in 38 eyes. The increase of $\geq$0.2 was associated with a worse prognosis in the visual field defect score (3.8±0.9 versus 1.0±0.3; p=0.0055, Mann-Whitney U test). Among the POAG eyes with high pressure, those with increases of C/D ratio $\geq$0.2 had higher average IOP levels than those with increases of <0.2 (17.6 ±2.1 mmHg versus 15.9 ±2.1 mmHg; p=0.017, Mann-Whitney U test). However, there was no significant difference between the average IOP levels of the two groups of NTG eyes (14.4 ±1.7 mmHg; $\geq$ 0.2 versus 14.2 ±1.3 mmHg; < 0.2; p=0.91, Mann-Whitney U test).