Potent Inhibition of Cicatricial Contraction in Proliferative Vitreoretinal Diseases by Statins

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Objective: In spite of tremendous progress in vitreoretinal surgery, certain post-surgical complications limit the success in the treatment of proliferative vitreoretinal diseases (PVD), such as proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR). One of the most significant complications is the cicatricial contraction of proliferative membranes, resulting in tractional retinal detachment and severe vision loss. Novel pharmaceutical approaches are thus urgently needed for the management of these vision-threatening diseases. In the current study, we investigated the inhibitory effects of statins on the progression of PVD.

Research Design and Methods: Human vitreous concentrations of TGF-β2 were measured by ELISA. TGF-β2- and vitreous-dependent phosphorylation of myosin light chain (MLC), a downstream mediator of Rho-kinase pathway, and collagen gel contraction simulating cicatrical contraction were analyzed using cultured hyalocytes. Inhibitory effects of simvastatin on cicatrical contraction were assessed both in vitro and in vivo.

Results: Human vitreous concentrations of TGF-β2 were significantly higher in the samples from patients with PVD compared to those with non-PVD. Simvastatin inhibited TGF-β2-dependent MLC phosphorylation and gel contraction in a dose- and time-dependent manner, and was capable of inhibiting translocation of Rho protein to the plasma membrane in the presence of TGF-β2. Vitreous samples from patients with PVD enhanced MLC phosphorylation and gel contraction, while simvastatin almost completely inhibited these phenomena. Finally, intravitreal injection of simvastatin dose-dependently prevented the progression of diseased states in an in vivo model of PVR.

Conclusions: Statins might have therapeutic potential in the prevention of PVD.
Proliferative vitreoretinal diseases (PVD), such as proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR), are common causes of severe vision loss (1). Surgical approaches for the treatment of these diseases have evolved significantly in the recent past, but the occurrence of postoperative complications, such as cicatricial contraction, limit the therapeutic success (2). Therefore, there is an urgent need for alternate pharmacological treatments of PVD that can complement, or potentially replace surgical intervention. In PDR and PVR, excessive wound healing and fibrosis induce the formation of proliferative membranes on the retinal surface. The proliferative membrane then extends into the vitreous and contracts, causing tractional detachment (3). The proliferative membrane consists of various cells, including hyalocytes, retinal pigment epithelial cells, glial cells and fibroblast-like cells (4-7).

Hyalocytes morphologically resemble macrophages, and are considered to originate from peripheral blood monocytes (8). Under physiological conditions, hyalocytes are mainly located in the cortical vitreous and are considered to maintain its transparency (9; 10). Under pathologic conditions, hyalocytes are thought to be critical in vitreoretinal interface diseases, such as idiopathic epiretinal membrane formation, macular hole (MH), and diabetic macular edema (11). Hyalocytes in diabetic eyes are higher in number and of different shape compared to those in normal eyes (12).

Transforming growth factor-β (TGF-β) is pivotal to tissue fibrosis. Among the three isoforms of TGF-β, TGF-β2 is the predominant isoform in the vitreous (13; 14). We and others have shown that TGF-β2 is over-expressed in the epiretinal membrane and vitreous of PDR and PVR patients, and that its expression correlates with the presence of intraocular fibrosis (14-17). TGF-β2 modulates the differentiation of various cell types, and is considered to increase the production of extracellular matrix, resulting in the formation and contraction of proliferative membranes (18; 19). Thus, it is possible that the combination of hyalocytes and TGF-β2 may contribute to the pathogenesis of PVD.

Statins, inhibitors of the 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase, are widely used to reduce endogenous cholesterol synthesis and improve hypercholesterolemia (20). HMG-CoA reductase is an upstream enzyme in the mevalonate biosynthetic pathway that catalyzes the conversion of HMG-CoA into mevalonate and then, in a number of steps, into farnesylpyrophosphate (FPP), a precursor of cholesterol (21). By inhibiting HMG-CoA reductase, statins block the mevalonate pathway, resulting in reduced synthesis of FPP and cholesterol. Statins are divided into 3 categories: the natural (i.e. lovastatin (LS) and pravastatin (PS)), the semi-synthetic (i.e. simvastatin (SS)) and the synthetic (i.e. atorvastatin, fluvastatin (FS) and cerivastatin (CS)). Statins decrease the risk for cardiovascular events even among high-risk individuals with coronary disease and diabetes (22). Moreover, statins may be useful in the treatment of other conditions, such as osteoporosis (23) and cancer growth and metastasis (24; 25).

Another product of the mevalonate pathway is geranylgeranylpyrophosphate (GGPP) that is synthesized from FPP. Both FPP and GGPP are important isoprenoid intermediates and serve as lipid attachments for a variety of intracellular proteins to the plasma membrane, including the γ subunit of heterotrimeric G proteins and the small GTP-binding proteins such as Ras and Rho, resulting in their activation (26). Rho translocation from the cytoplasm to the plasma membrane is dependent on geranylgeranylation (GGPP attachment), whereas Ras translocation is dependent on
farnesylation (FPP attachment) (27). Rho in the plasma membrane is implicated in the cytoskeletal responses to extracellular signals, and is converted to an active GDP-bound state (28). Rho-kinase, one of the effector molecules of Rho, is involved in a variety of cellular events related to cell morphology, adhesion and motility (28-30), especially through phosphorylation of the myosin light chain (MLC). MLC phosphorylation induces actin-myosin interaction, and consequently smooth muscle contraction and stress fiber formation in non-muscle cells (31; 32). Activation of the Rho/Rho-kinase pathway is therefore indispensable for smooth muscle contraction. GGPP is one of the downstream components of the mevalonate pathway and plays an important part in the Rho/Rho-kinase pathway activation. Thus, statins, which regulate the mevalonate pathway, also regulate the Rho/Rho-kinase pathway and consequently MLC phosphorylation.

Previously, we reported that the Rho-kinase pathway is involved in TGF-β2-induced MLC phosphorylation and contraction of hyalocyte-containing collagen gels, and that hydroxyfasudil, a potent Rho-kinase inhibitor, significantly diminishes these TGF-β2-induced effects (18). In the present study we investigate the regulatory effects of statins on TGF-β2- and vitreous-dependent MLC phosphorylation and contraction of hyalocyte-containing collagen gels, and its therapeutic potential for prevention of PVD in vivo.

**RESEARCH DESIGN AND METHODS**

**Reagents.** Simvastatin (SS), fluvastatin (FS), pravastatin (PS) and cerivastatin (CS) were purchased from CalBiochem (CA, USA). Lovastatin (LS) was purchased from Funakoshi Corporation (Tokyo, Japan). SS was used as an active form after treatment with NaOH.

**Vitreous samples and Enzyme-linked immunosorbent assay.** This study was carried out with approval from the Institutional Review Board and performed in accordance with the ethical standards of the 1989 Declaration of Helsinki. We obtained written informed consent to participation from the patients. Vitreous samples were collected from patients who underwent pars plana vitrectomy due to non-PVD (MH) or PVD (PDR and PVR). Concentrations of TGF-β2 were measured by a human TGF-β2 immunoassay kit (R&D Systems, MN, USA).

**Cell culture.** Bovine hyalocytes were isolated as we previously reported (33). Cultured hyalocytes obtained between passages 5 to 7 were used in experiments.

**Collagen gel contraction assay.** The contraction assay was performed as we previously described (33). Type I collagen (Koken Co., Ltd., Tokyo, Japan), a reconstitution buffer, hyalocytes suspension, and distilled water were mixed, and added to a 24-multiwell plate (Nunc, Roskilde, Denmark). After 1 hour’s pretreatment of vitreous samples with anti-TGF-β2 antibody (R&D Systems) (1 μg/ml) or mouse IgG (Sigma-Aldrich) (1 μg/ml) that was added for confirming the absence of non-specific suppression of the gel contraction by anti-TGF-β2 antibody, the gels were treated with the vitreous samples (400 μl). The diameter of the collagen gel was measured at 5 days after the treatment. For quantitative purposes, contraction data is presented as the reduction in diameter of the collagen gels.

In the same way, collagen gels containing hyalocytes were starved, pretreated with statins for 24 hours, and then treated with TGF-β2 (Sigma-Aldrich) (3 ng/ml) or vitreous samples.

**Western blot analysis.** Total cell lysates were subjected to 15% SDS-PAGE and the blots were incubated with an antibody against phosphorylated-MLC (p-MLC; Santa Cruz, CA, USA) (1:1000). Visualization was performed with an enhanced chemiluminescence (ECL; Amersham, IL, USA) detection system. Lane loading
differences were normalized by reblotting the membranes with an antibody against MLC (Santa Cruz) (1:1000).

Plasma membrane proteins from the cells were isolated by Qproteome Plasma Membrane Protein Kit (QIAGEN, Hilden, Germany), and were subjected to Western blot analysis with an antibody against Rho (Upstate, VA, USA) (1:1000).

**Counting viable cells in collagen gels.** After five days’ treatment, the collagen gels were dissolved by collagenase and the cell suspension was collected. The viable cell number was counted with hemocytometer after trypan blue staining.

**In vivo model of PVR.** All experimental procedures using animals adhered to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Ophthalmic and Vision Research. We caused experimental PVR in rabbit eyes as previously described (34). Homologous conjunctival fibroblasts were prepared by harvesting the conjunctival tissue. Twenty eyes from twenty Dutch rabbits weighing 2 to 2.5 kg were assigned into three groups, and fibroblasts (100,000 cells) were injected into the right eye in each rabbit. Eyes in first group (6 eyes) were administered an intravitreal injection of 0.1 ml vehicle (balanced salt solution). Eyes in second group (7 eyes) were administered an intravitreal injection of 0.1 ml vehicle containing SS (5 μM final intravitreal concentration). Eyes in last group (7 eyes) were administered an intravitreal injection of 0.1 ml vehicle containing SS (15 μM final intravitreal concentration). Clinical observations were performed carefully as long as 28 days after surgery and categorized according with the PVR classification of Fastenberg et al (35).

**Electroretinography (ERG).** Nine eyes from ten Dutch rabbits weighing 2 to 2.5 kg were assigned into three groups. Eyes in the first group (3 eyes) were administered an intravitreal injection of 0.1 ml vehicle. Eyes in the second group (3 eyes) were administered an intravitreal injection of 0.1 ml vehicle containing SS (5 μM final intravitreal concentration). Eyes in the third group (3 eyes) were administered an intravitreal injection of 0.1 ml vehicle containing SS (15 μM final intravitreal concentration). Electroretinogram was performed on day 28 as previously described (36).

**Light microscopy.** The eyes were enucleated and fixed in 4% paraformaldehyde on day 28. Whole eyes were cut approximately along the vertical meridian. Paraffin-embedded sections were stained with hematoxyline-eosin (HE) and each section was examined using light microscopy.

**TdT-dUTP terminal nick-end labeling (TUNEL) assay.** Apoptotic and potentially necrotic cell death was detected by TUNEL. The eyes were fixed in 4% paraformaldehyde and embedded in paraffin. TUNEL staining was performed with the TdT Fluorescein in situ apoptosis detection kit (R&D Systems), according to the manufacturer’s protocols. The sections were co-stained with propidium iodide (Molecular Probes, Eugene, OR) to observe the cell nuclei by fluorescence microscopy (KEYENCE, BIORAVO, BZ-9000). As a TUNEL positive-control, we used the retina of rabbit PVR model on day 7 after the onset.

**Transmission electron microscopy (TEM).** The eyes were fixed in 1% glutaraldehyde and 1% paraformaldehyde in PBS. The specimens were postfixed in veronal acetate buffer osmium tetroxide (2%), dehydrated in ethanol and water, and embedded in Epon. Ultrathin sections were cut from blocks and mounted on copper grids. The specimens were observed with an electron microscope (H7650, Hitachi, Japan).

**Statistical analysis.** Statistical differences were assessed using nonparametric test
(Kruskal-Wallis variance analysis) for the data of TGF-β2 concentrations, and ANOVA for the other groups. P values less than 0.05 were considered significant. To adjust for inflated α error due to multiple comparisons, the corrected significant P value was defined using the Bonferroni correction.

RESULTS

TGF-β2 in the vitreous and its impact on membrane contraction. The median TGF-β2 protein concentrations, 2.35 ng/ml (range 0.72-5.26) in MH (n=27), 4.74 ng/ml (range 1.60-13.0) in PDR (n=53), and 4.48 ng/ml (range 2.52-21.6) in PVR (n=22) patients, were significantly higher in PDR or PVR patients than in patients with MH (p<0.01) (Fig. 1A). The median TGF-β2 protein concentration in the vitreous samples from patients with PDR or PVR showed no significant difference (p=0.6).

Vitreous samples from patients with PVD and those containing non-specific IgG induced significant contraction of hyalocyte-containing collagen gels (63% and 67% vs. control, respectively). In comparison, the vitreous-induced contraction was virtually suppressed by specific anti-TGF-β2 antibody (p<0.05) (97% vs. control) (Fig. 1B, C), suggesting a key role for TGF-β2 in the vitreous-induced collagen gel contraction.

Role of SS in TGF-β2-dependent MLC phosphorylation. TGF-β2 enhanced MLC phosphorylation about 2 times compared with control (Fig. 2A, B). TGF-β2-dependent MLC phosphorylation showed a significant reduction at 0.3 μM SS or higher concentrations (up to 10 μM SS) compared to TGF-β2 alone (p<0.05). The level of MLC phosphorylation at 10 μM SS concentration was lower than in untreated control, suggesting that SS at higher concentrations may block the constitutive level of MLC phosphorylation. Since 3 μM SS was sufficient to reverse the effect of TGF-β2 on MLC phosphorylation, we chose the slightly higher concentration of 5 μM to study the time-dependent effect of SS on TGF-β2-dependent MLC phosphorylation. Treatment of the hyalocytes with 5 μM SS for 1 hour or longer (up to 24 hours) significantly suppressed the MLC phosphorylation compared to TGF-β2 alone, and treatment with 5 μM SS for 24 hours sufficiently suppressed the MLC phosphorylation below that of untreated control (Fig. 2C, D).

TGF-β2-dependent collagen gel contraction and its inhibition by SS. The control gels showed no apparent contraction up to 5 days, whereas TGF-β2 caused substantial collagen gel contraction in a time-dependent manner, in the first 5 days (57.6% vs. control). TGF-β2-dependent collagen gel contraction was significantly reduced by SS starting at a concentration of 1 μM, and the reduction was greater at 3 and 10 μM (92% and 100% vs. control, respectively) (Fig. 3).

TGF-β2-dependent Rho translocation to the plasma membrane and its inhibition by SS. TGF-β2 significantly enhanced the Rho translocation to the plasma membrane (p<0.05), while SS suppressed the translocation (Fig 4A, B). However, the effect of SS was reversed in the presence of mevalonate (400 μM), a component of the mevalonate pathway. These findings suggest that SS inhibits Rho/Rho kinase pathway by preventing Rho from translocating to the plasma membrane via inhibition of the mevalonate pathway.

Comparison of the effects of various statins on TGF-β2-dependent MLC phosphorylation and collagen gel contraction. SS and FS significantly suppressed TGF-β2-dependent MLC phosphorylation by hyalocytes, while PS did not show an effect (Fig. 5A, B). In addition, SS significantly suppressed MLC phosphorylation compared with FS (p<0.05). Next, we compared the inhibitory effect of statins on the contraction of hyalocyte-containing collagen gels. SS and FS
significantly suppressed TGF-β2-dependent contraction of collagen gel, while PS did not show an effect (Fig. 5C, D). In addition, the inhibitory effect of SS (100% vs. control) was significantly higher than that by FS (94% vs. control) (p<0.05). To test whether cytokine and/or statin treatment affected the growth and/or viability of the cells in our experiments, we treated the collagen gels with collagenase and counted the number of the viable cells. Both TGF-β2 and statins showed no significant effects on cell number in the three dimensional collagen gels (Fig. 5E).

**Impact of SS on MLC phosphorylation and collagen gel contraction induced by vitreous samples from patients with PVD.** Vitreous samples from patients with PVD caused a significantly larger enhancement of MLC phosphorylation than those from patients with non-PVD (Fig. 6A, B). SS (5 μM) strongly attenuated the vitreous-induced MLC phosphorylation. Expression of GAPDH did not change after the treatment with SS or stimulation with vitreous samples. However, total MLC increased in cells stimulated with the vitreous samples compared to cells without vitreous treatment, and the increase was larger in cells stimulated with vitreous samples from patients with PVD than those from patients with non-PVD. These increases in an amount of total MLC were suppressed by 5 μM SS.

The contraction of hyalocyte-containing collagen gels stimulated with the vitreous samples from patients with PVD was significantly larger compared to the contraction of the gels stimulated with vitreous samples from patients with non-PVD (p<0.05) (Fig. 6C, D). We further examined the inhibitory effect of SS on vitreous-dependent contraction of collagen gels, and found that 5 μM SS suppressed the collagen gel contraction induced by vitreous samples even from patients with PVD.

**SS inhibition of PVR development in vivo.** The control eyes of rabbits injected with vehicle developed PVR and were accompanied by proliferative membrane formation and cicatricial contraction, resulting in tractional retinal detachment (Fig. 7A). In comparison, 5 and 15 μM SS (final intravitreal concentrations) significantly prevented PVR development (Fig. 7C). SS inhibited the contraction of the proliferative membrane, and the membranes in eyes treated with SS were much thinner than those in vehicle treated eyes (Fig. 7B). After SS injections were stopped at day 7, the eyes treated with 5 μM SS developed a mild but significant PVR, while no significant PVR development was observed in the eyes treated with 15 μM SS.

**Absence of apparent adverse effects of SS in the retina.** To evaluate the retinal function after intravitreal application of SS, we performed ERG. The mean amplitude and latency of the 2Hz b wave in the eyes treated with 5 or 15 μM SS were 203.4 μV and 28.1 ms, and 201.7 μV and 28.4 ms, not significantly different from those without any treatment or those of the vehicle treated eyes (204.8 μV and 28.1 ms), suggesting that at these concentrations SS may not impede retinal function (Fig. 8A).

The histological structure of the retina in the eyes injected with SS appeared normal when observed on day 28 (Fig. 8B, C). In the retina of an experimental PVR as a positive-control, apoptotic cells, which present TUNEL-positive staining (green), were detected in the inner nuclear layer and the outer nuclear layer (Fig. 8D). The eyes injected with SS had no apparent TUNEL-positive staining in the retina when observed on day 28 (Fig. 8E).

The eyes injected with SS had no apparent ultrastructural changes in internal limiting membrane, nerve fiber layer and ganglion cell layer (Fig. 8G) compared to the untreated control eyes (Fig. 8F). In other parts of the retina, such as inner and outer nuclear layers, photoreceptors, and retinal pigment
epitheliums, there were also no apparent changes.

DISCUSSION
Blindness is a devastating consequence of PVD such as PDR and PVR. Currently, the progression of these diseases cannot be effectively prevented and the treatment options are limited to vitreoretinal surgery. An effective pharmacological treatment is thus urgently needed to complement or potentially replace the surgical intervention. In the current study, we show statins’ novel function in inhibiting the Rho/Rho-kinase pathway, the contraction of collagen gel, and the progression of experimental PVR, suggesting the therapeutic potential of these compounds for the treatment of PVD.

Various cytokines, such as TGF-β, connective tissue growth factor (CTGF), interleukin-6 (IL-6) and platelet-derived growth factor (PDGF) are over-expressed in the vitreous and membranes associated with PDR and PVR, and they contribute to the pathogenesis of these diseases (15; 37-40). Among these cytokines, TGF-β2 induces the transformation of retinal pigment epithelial cells or hyalocytes to myofibroblastic cells (18; 41), and plays a key role in the formation and contraction of proliferative membranes. In this study, we confirmed the overexpression of TGF-β2 in the vitreous from patients with PVD (PDR and PVR), and showed that TGF-β2 inhibition strongly suppressed the vitreous-induced contraction of the collagen gels. This indicates the possibility that TGF-β2 is the dominant contributor to the contraction of proliferative membrane in the vitreous cavity. Thus, we focused on the role of TGF-β2 to investigate the mechanisms of membrane contraction. TGF-β2 enhanced MLC phosphorylation in hyalocytes that was responsible for the contraction of the hyalocyte containing collagen gels. We showed that SS suppressed TGF-β2-induced MLC phosphorylation and collagen gel contraction in a dose-dependent fashion by inhibiting GGPP-mediated translocation of Rho to the plasma membrane, while no signs of cytotoxicity were apparent. Differences in statin’s structural characteristics cause different levels of lipophilicity, and possibly of efficacies and also cytotoxicity. PS is strongly hydrophilic, and SS is much more lipophilic than PS (42). FS is also more lipophilic than PS, however less than SS (43). While our comparison of the inhibitory effects of various statins, SS, FS and PS, revealed that SS was more effective in reducing MLC phosphorylation than the other statins under the chosen experimental conditions, the results might vary at other time points or concentrations. Further examination is necessary to determine the order. Additionally, since CS is more lipophilic than SS (44), we studied the effects of CS and SS. Many hyalocytes treated with CS (5 μM for 24 hours) shrank and detached from the culture plates, while the cells treated with SS (5 μM) remained morphologically unchanged. However, at higher concentrations (>20 μM), some hyalocyte shrinkage was observed even with SS (data not shown). CS is known to be cytotoxic and induce apoptosis (45). The morphological changes we observed in CS-treated hyalocytes in our study suggest that this drug may also be toxic to hyalocytes and promote their apoptosis. Since the lipophilicity of LS is similar to that of SS (42), we compared their effect and found the inhibitory effect of SS on MLC phosphorylation and collagen gel contraction to be greater than that of LS (5 μM for 24 hours) (data not shown).

Vitreous samples from patients with PDR and PVR induced MLC phosphorylation and contraction of hyalocyte-containing collagen gels, which were effectively inhibited by SS. It may appear surprising that not only vitreous samples from patients with PVD, but also those from non-PVD patients induced MLC phosphorylation and collagen gel contraction. However, this might be explained by the fact
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that TGF-β2 is expressed in vitreous samples of patients with MH at high enough concentrations to induce the observed phenomena. The concentrations of TGF-β2 in vitreous samples from patients with PVD (PDR and PVR) were higher than in those from MH patients. Therefore, in a concentration-dependent manner, the inductions might be greater in the vitreous samples from patients with PVD than in those from patients with non-PVD. The concentrations of TGF-β2 in some vitreous samples from patients with MH were higher than those with PDR and PVR. However, the pathology of MH does not generally involve proliferative membrane formation and tractional retinal detachment. Occasionally epiretinal membranes and retinal folds accompany MH. Retinal folds are considered to be caused by the contraction of the epiretinal membranes. TGF-β2 concentrations in vitreous samples from patients with MH may be high enough to induce contraction of proliferative membranes, however, since the epiretinal membrane in these patients does not extend into the vitreous and is very thin, the expression of TGF-β2 remains inconsequential.

MLC phosphorylation depends on the concentration of TGF-β2 (18), thus the eyes with PVR having lower TGF-β2 concentrations might represent a less severe pathology, or remission, compared to those with higher TGF-β2 concentrations. The occurrence of tractional retinal detachment might depend on the presence or absence of proliferative membranes and the concentration of TGF-β2.

While TGF-β2 stimulated hyalocytes showed no significant change in MLC expression, those stimulated with vitreous samples showed elevated MLC expression that was inhibited by SS treatment. The vitreous includes various cytokines, released from retinal pigment epithelial cells, glial cells, macrophages and other intravitreal cells (46). Thus, some cytokines other than TGF-β2 might elevate the expression of MLC. Among the cytokines found in the vitreous, insulin like growth factor 1 (IGF-1), PDGF and members of the endothelin family have been shown to stimulate extracellular matrix contraction (18; 47; 48). This study reveals a key role for TGF-β2 in the pathology of PVD, however it is possible that other cytokines found in the vitreous might also be involved in MLC phosphorylation and contraction of hyalocyte-containing collagen gels. Since SS almost completely inhibited the phenomena induced by vitreous samples, it might also inhibit the effect of the other cytokines such as IGF-1, PDGF and members of the endothelin family and unknowns, which might be exerted through the Rho/Rho-kinase pathway.

SS also prevented the development of PVR in vivo. Proliferative membranes in SS injected eyes, even if present, were very thin, while the membranes in vehicle injected eyes with PVR in stage 4 or 5 were thick. Thus, SS might have also an inhibitory effect on the formation and growth of proliferative membranes in addition to the cicatricial contraction of proliferative membranes. However, after the end of the SS injections, even in the groups treated with higher concentration of SS (15 µM) the development of PVR was not completely inhibited. This may be due to a short biological half-life time of the compound in the vitreous cavity. Therefore, to sustain a constant level of intravitreal SS concentration, frequent injections or a slow release drug delivery system might be necessary.

Recent findings suggest that statins might have a number of beneficial effects in the eye. While the applicability of statins on age-related macular degeneration is still under investigation (49), statins are shown to have protective effects on primary open-angle glaucoma, a major cause of blindness (50).
The regulation of the intraocular pressure (IOP) within a physiological range is of great clinical importance. Statins are reported to down-regulate the IOP by increasing aqueous humor outflow via the inhibition of Rho/Rho-kinase pathway in the trabecular meshwork and the ciliary body (50).

In conclusion, SS potently inhibits the Rho/Rho-kinase pathway and thus might have therapeutic potential in the prevention of cicatricial contraction of proliferative membranes in vivo. This is the first report demonstrating the beneficial effects of SS in inhibiting the development of PVR. Statins might provide a new strategy for the treatment and prevention of the development of PVD in human.

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Figure 1. TGF-β2 expression in the vitreous. (A) Vitreous samples were collected from patients with non-PVD (MH) and PVD (PDR and PVR). Concentrations of TGF-β2 in the vitreous were measured by enzyme-linked immunosorbent assay (MH, n=27; PDR, n=53; PVR, n=22). **p<0.01 compared with MH. (B) Hyalocytes were embedded in type I collagen gels (n=4). After starvation and pretreatment with anti-TGF-β2 antibody (1 μg/ml) or IgG (1 μg/ml) for 1 hour, the collagen gels were treated with vitreous samples from patients with PVD. Five days after the treatment, the gels were photographed. (C) The diameter of the collagen gels was measured and expressed as percentage of the average diameter of the control group. *p<0.05; NS, not significant.
Figure 2. Inhibitory effect of SS on TGF-β2-dependent MLC phosphorylation. Hyalocytes were starved in DMEM containing 3% calf serum for 24 hours. (A) Hyalocytes were pretreated for 30 minutes with or without the indicated concentrations of SS (0.3, 1, 3, and 10 μM), and subsequently treated with 3 ng/ml of TGF-β2 for 24 hours. Total cell lysates were subjected to Western blot analysis with an antibody against p-MLC. Lane loading differences were normalized by reblotting the membranes with an antibody against MLC. (C) Hyalocytes were pretreated with or without 5 μM of SS for the indicated time (1, 4, 10, and 24 hours), and subsequently treated with 3 ng/ml of TGF-β2 for 24 hours. p-MLC and MLC were also examined in the same way as in Fig 1A. (B, D) Signal intensity ratios (p-MLC to MLC) were expressed as percentage of control intensity ratio. *p<0.05 compared with TGF-β2 alone.
Figure 3. The effect of SS on TGF-β2-induced contraction of hyalocyte-containing collagen gels. Hyalocytes were embedded in type I collagen gels (n=4). (A) After starvation and pretreatment with indicated concentrations of SS for 24 hours, the collagen gels were stimulated with TGF-β2 (3 ng/ml). Five days after the stimulation, the gels were photographed. (B) The diameter of the collagen gels was measured and expressed as a percentage of the average diameter of control group. *p<0.05; NS, not significant compared with TGF-β2 alone.
Figure 4. Inhibitory effect of SS on the Rho translocation to the plasma membrane. (A) Hyalocytes were pretreated with SS (5 μM) with or without mevalonate (Mev), and then treated with TGF-β2 (3 ng/ml) for 24 hours. Plasma membrane proteins were isolated and subjected to Western blot analysis with an antibody against Rho. (B) Signal intensities were expressed as percentage of control intensity. *p<0.05; NS, not significant.
Figure 5. Comparison of inhibitory effects of SS, FS and PS on TGF-β2-dependent MLC phosphorylation and collagen gel contraction. (A) Starved hyalocytes were pretreated with vehicle, 5 μM of SS, FS or PS for 30 minutes, and subsequently treated with or without 3 ng/ml of TGF-β2 for 24 hours. Total cell lysates were subjected to Western blot analysis with an antibody against p-MLC. Lane loading differences were normalized by reblotting the membranes with an antibody against MLC. (B) Signal intensity ratios (p-MLC to MLC) were expressed as percentage of intensity ratio of vehicle alone. *p<0.05; NS, not significant. (C) Hyalocytes were embedded in type I collagen gels (n=4). After starvation and pretreatment with vehicle, 5 μM of SS, FS or PS for 24 hours, the collagen gels were stimulated with TGF-β2 (3 ng/ml). Five days after the stimulation, the gels were photographed. (D) The diameter of the collagen gels was measured and expressed as percentage of the average diameter of control group. **p<0.01, *p<0.05; NS, not significant. (E) The viable cell number in the collagen gels was counted to exclude the effect of cell growth or cytotoxicity on the collagen gel contraction or its inhibition. Five days after the treatment, the collagen gels were dissolved and the cell suspension was collected. The viable cell number was counted with hemocytometer after trypan blue staining. (n=4; NS, not significant compared with control).
**Figure 6.** Inhibitory effects of SS on vitreous-induced MLC phosphorylation and contraction of hyalocyte-containing collagen gels. (A) Starved hyalocytes were pretreated with 5 μM of SS for 30 minutes, and subsequently treated with or without 400 μl vitreous samples of non-PVD (MH) or PVD (PDR and PVR) for 24 hours. Total cell lysates were subjected to Western blot analysis with an antibody against p-MLC. Lane loading differences were normalized by reblotting the membranes with an antibody against MLC and GAPDH. (B) Signal intensity ratios (p-MLC to GAPDH) were expressed as percentage of control intensity ratio. (C) Hyalocytes were embedded in type I collagen gels (n=4). After starvation and pretreatment with 5 μM of SS for 24 hours, the collagen gels were stimulated with 400 μl vitreous samples of non-PVD or PVD. Five days after the stimulation, the gels were photographed. (D) The diameter of the collagen gels was measured and expressed as percentage of the average diameter of the control group. *p<0.05.
Figure 7. Inhibitory effects of SS on experimental PVR in rabbit eyes. Rabbits underwent vitrectomy and intravitreal injection of fibroblasts with and without SS on day 0. The eyes were injected with the same contents of SS on days 1, 3, 5, and 7, and examined using indirect ophthalmoscope as long as 28 days after the surgery. (A) A representative vehicle-injected eye with PVR in stage 5, 28 days after the surgery. Proliferative membranes were observed in the vitreous cavity, causing a traction to the retina and retinal detachment. (B) A representative SS-injected eye (15 μM) with PVR in stage 1. A thin proliferative membrane was observed, however there was no evidence of tractional retinal detachment. (C) Clinical observations were categorized according to the PVR classification of Fastenberg et al (35). Stage 1, intravitreal membrane, stage 2, focal traction, localized vascular changes, hyperemia, engorgement, dilation, blood vessel elevation, stage 3, localized detachment of medullary ray, stage 4, extensive retinal detachment, total medullary ray detachment, peripapillary retinal detachment, and stage 5, total retinal detachment. Black circles=vehicle; white squares=5 μM SS; black triangle=15 μM SS. **p<0.01, * p<0.05; NS, not significant compared with vehicle.
Figure 8. Histological and physiological examinations of eyes injected with SS. Rabbits received intravitreal injections of 0.1 ml vehicle or vehicle with SS (final concentration of 5 μM or 15 μM SS) days 0, 1, 3, 5 and 7. (A) Electroretinograms on day 28. A flash stimulus of intensity 1.30 log cd.s/m² was superimposed on a background luminance of 1.15 log cd/m². Light microscopy of the rabbit eye without any treatment (B) and that of the eye injected with SS (15 μM) (C). Scale bar 100 μm. Apoptotic and potentially necrotic cell death detected by TUNEL in the section from positive-control (rabbit PVR model on day 7 after its onset) (D) and in the section from the SS (15 μM) treated eye (E). Scale bar 50 μm. Transmission electron microscopy of the rabbit eye without treatment (F) and that of the eye injected with SS (15 μM) (G). Scale bar 10 μm.