Involvement of Rho-Kinase Pathway in Contractile Activity of Rabbit RPE Cells In Vivo and In Vitro

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Purpose. Increased α-smooth muscle actin (α-SMA) expression in epiretinal membranes causes tractional retinal detachment (TRD) in proliferative vitreoretinopathy (PVR). The Rho-A/Rho–associated kinase signaling pathway is a principal mediator of contractile force generation in nonmuscle cells. In the current study, the relation between the Rho-kinase pathway and α-SMA expression and type I collagen gel contractile activity in retinal pigment epithelial (RPE) cells was investigated, using Y27632, a specific inhibitor of p160ROCK, and the involvement of the Rho-kinase pathway was evaluated in a rabbit PVR model with cultured RPE cells and platelet-rich plasma (PRP).

Methods. RPE cells were obtained from rabbits and cultured. The number of passages and the effect of Y27632 on α-SMA expression were studied by immunohistochemistry and immunoblot analysis. An in vitro type I collagen gel contraction assay and MTT assay evaluated the effect of Y27632 on RPE cell contractile force and proliferation. Cultured sixth-passage rabbit RPE cells were coinjected with PRP intravitreally, followed by 50 μM of Y27632, injected weekly. The presence of TRD was assessed until 28 days to evaluate the effect of Y27632 in vivo.

Results. Expression of α-SMA was increased according to the passages. Y27632 suppressed α-SMA expression in cultured RPE cells and impaired contractile force. Y27632 did not affect the proliferative potential. Y27632 significantly ($P < 0.01$) reduced TRD development.

Conclusions. Y27632 decreased α-SMA expression and the contractile force generated by RPE cells and attenuated PVR, indicating the involvement of the Rho-kinase pathway in cell-dependent collagen contraction in vitro and in vivo. The drug may affect the biological event by inhibiting α-SMA expression, and Y27632 could be useful for preventing PVR. (*Invest Ophthalmol Vis Sci.* 2004;45:668–674) DOI:10.1167/iovs.02-0808

Contraction of epiretinal membranes (ERMs) remains the leading cause of failure in retinal surgery.[1,2] In patients with proliferative vitreoretinopathy (PVR), ERMs are characterized by the diffuse presence of α-smooth muscle actin (α-SMA)–positive myofibroblasts,[3] the origin of which is uncertain and is presumed to be dedifferentiated RPE cells.[4,5] α-SMA, a highly conserved protein and major component of microfilaments that control cell morphology and motility, is also a sensitive marker of RPE phenotype change.[5–7] In RPE cells, α-SMA is upregulated by transforming growth factor-(TGF)-β, which is believed to play a main role in the development of PVR.[8,9] Recently, dense bundles of α-SMA microfilaments forming stress fibers within the myofibroblast were observed by electron microscopy in the ERM of patients with PVR,[10] which strongly suggests that α-SMA substantially contributes to PVR development.

Contraction of ERMs is a cell-mediated event,[11–13] which may be involved with two possible mechanisms: one is active contraction of myofibroblasts[14,15] and the other based on the motile activity of myofibroblasts that causes remodeling of the surrounding extracellular matrix.[16] The NH2-terminal peptide of α-SMA was reported to inhibit contractile force causing retinal detachment,[17] and an antagonist of α-SMA should therefore prevent development of PVR.

A small GTPase, Rho, regulates the organization of the actin cytoskeleton by promoting the assembly of focal adhesions and actin stress fibers.[18–20] Several putative Rho target molecules have been isolated.[21] Among them, a family of Rho-associated serine/threonine kinase isozymes named p160ROCK[22] and ROK_{α}/Rho-kinase/ROCK-II[23,24] has been identified as a new class of Rho effectors that can induce focal adhesions and stress fibers in cultured fibroblasts and epithelial cells.[25] It is now known that actomyosin contraction can be regulated by Rho-kinase in two ways, one of which is by phosphorylating myosin regulatory light chain.[26,27] The other way is by inhibiting the myosin phosphatase activity through phosphorylation of myosin-bound subunit (MBS) of myosin phosphates.[28]

Y27632 is a new pyridine derivative that acts as a specific inhibitor of the ROCK/ROK family of protein kinases.[29] Y27632 inhibits smooth muscle contraction both in vitro and in vivo as well as the formation of stress fibers and focal adhesions induced by p160ROCK in cultured cells. This compound prevents hepatic fibrosis[30] and glaucoma[31] and blocks both Rho-mediated activation of actomyosin and invasive activity of cancer cells in vitro without toxicity.[32,33] We studied the effect of Y27632, a specific p160ROCK inhibitor, on RPE-mediated cellular contraction in vitro and in vivo.

Materials and Methods

Rabbit RPE Cell Culture

Rabbit RPE cell cultures were obtained from pigmented Dutch rabbits by the method of Flood et al.[34] with slight modification. Briefly, the freshly enucleated eyes were immediately submerged in the RPE medium consisting of Eagle's minimum essential medium (EMEM; Invitrogen-Gibco, Grand Island, NY), 10% (vol/vol) fetal bovine serum (FBS; BioSource International, Camarillo, CA), and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). The globes were opened and the cornea, lens, and vitreous humor were removed by a circumferential cut just posterior to the ora serrata. The neural retina was carefully washed out by the RPE medium. The eye cups were washed with Hank's balanced salt solution (HBSS) and digested with 0.012% (wt/

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vol) trypsin (Nacalai Tesque, Inc., Kyoto, Japan) in 0.005% (wt/vol) EDTA (Nacalai Tesque, Inc.) for 1 hour at 37°C. The trypsinization was stopped by adding excess RPE medium. The dissociated RPE cells were carefully washed out, without disturbing the underlying choroids. RPE cells were first cultured in 12-well plates to near confluence with the RPE medium, and then were passed to 25 cm^2 flasks. The cells were trypsinized and passaged every week.

Trypan Blue Exclusion Test

To confirm the safety of the drug, the cultured RPE cells were exposed to Y27632, and the cytotoxicity was measured using the trypan blue exclusion test. Y27632 (*trans*-4-[(1*R*)-1-Aminoethyl]-*N*-4-piridinylcyclohexanecarboxamide dihydrochloride) was obtained from Torcris Cookson (Bristol, UK), and the manufacturer reported it to be more than 98% pure. Viable cells were counted in vitro according to the method previously described.[35] Briefly, the 5×10^5 RPE cells were plated in 100-mm dishes and grown in the RPE medium for 24 hours at 37°C. The medium was then replaced with regular media, with or without 10 or 50 μM of Y27632. Twenty-four hours after the treatment, the cells were trypsinized and 1 mL of cell suspension containing 2×10^6 RPE cells was prepared; 50 μL of 0.1% (vol/vol) trypan blue solution then was added to the resuspension. Stained and unstained cells were counted by hemocytometry under a microscope 3 minutes after the treatment. The percentage of cell viability was calculated using the following formula: % cell viability = (viable cell count/total cell count) $\times$ 100. Only cells from the sixth passage were used. Three independent experiments were performed.

Immunohistochemistry

Immunohistochemistry was performed according to a method previously described.[7] Briefly, RPE cells in passage 3 or 6 were plated on glass slides and grown at 37°C. Y27632 (10 and 50 μM) was added and incubated for the time indicated in Figures 3 and 4. For control experiments, phosphate-buffered saline (PBS) was added. Cells were washed with PBS three times and fixed with methanol for 10 minutes at −24°C. Then, RPE cells were blocked with PBS containing 20% (vol/vol) normal goat serum (The Binding Site, San Diego, CA) and 5% (wt/vol) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) for 1 hour in a moist chamber at room temperature after washing with PBS for 10 minutes. Thereafter, the cells were washed with PBS for 10 minutes three times and incubated with primary antibody at 4°C overnight. The primary antibody was monoclonal mouse anti-αSMA (clone 1A4; Sigma-Aldrich) and was omitted in negative control experiments. The cells were incubated for another 60 minutes with rhodamine-conjugated goat anti-mouse IgG (Sigma-Aldrich) after they were washed with PBS three times. Finally, cells were mounted and observed by fluorescence microscope (Axiovert; Carl Zeiss Meditec, Oberkochen, Germany). The primary antibody was diluted 1:800 and the secondary antibody was diluted 1:100 with the dilution buffer (PBS containing 1% [vol/vol] normal goat serum and 3% [wt/vol] BSA).

Immunoblot Analysis

Immunoblot analysis was performed according to a method previously described,[36] with slight modification. RPE cells (5×10^4 cells/well) were cultured in six-well culture plates (Corning Costar, Corning, NY). After reaching subconfluence, RPE cells were washed with PBS three times and incubated with various concentrations of Y27632 (10 and 50 μM) with RPE medium containing 0.1% (wt/vol) BSA for the time indicated up to 24 hours. After 6 or 24 hours or a recovery period of 24 hours after treatment, the cells were gently washed three times with PBS and lysed in 200 μL of lysis buffer (0.1% sodium dodecyl sulfate [SDS]in 0.1 M Tris-HCl [pH 7.4]). After cell lysates were boiled at 96°C for 5 minutes in sample buffer containing 0.1% SDS, 10 μg protein of each samples was subjected to 12.5% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated in a blocking solution (5% skim milk, 0.1% Tween-20, and 10 mM Tris-HCl, [pH 7.4]) for 60 minutes at room temperature to avoid a nonspecific immunoreaction. The membrane was incubated for 12 hours at 4°C with anti-α-SMA antibody (Sigma-Aldrich) diluted 1:2000 with blocking solution, and washed three times with a washing solution (0.1% Tween-20 in Tris-buffered saline) for 10 minutes at room temperature. It was then incubated for 60 minutes at room temperature with horseradish-peroxidase–conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:2000. After the membranes were washed three times with the wash solution for 10 minutes at room temperature, they were stained with enhanced chemiluminescence (ECL) Western blot detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). The visualized images were scanned with a chemiluminescence imaging analyzer (Alpha Innotech Corp., San Leandro, CA). The exposed areas were measured with NIH Image software (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Only cells from the third and sixth passages were used in the experiment.

To confirm the amount of sample, the gel was stained with Coomassie brilliant blue to visualize the protein bands with a standard protocol. For staining with Coomassie blue, the gel was soaked in 0.2% Coomassie brilliant blue R-250 (Bio-Rad), 10% (vol/vol) acetic acid, and 45% (vol/vol) methanol for 20 minutes and then destained with 10% (vol/vol) acetic acid and 10% (vol/vol) methanol.

Type I Collagen Gel Contraction Assay

The contraction assay, previously described,[37] was performed with slight modifications. Cells were suspended in 1.5 mg/mL neutralized collagen I (Cohesion Vitrogen 100; Invitrogen, Palo Alto, CA) at a density of 10^6 cells/mL and transferred into a 24-well plate (Falcon, Franklin Lakes, NJ) that had been preincubated with a solution of PBS and 5 mg/mL BSA overnight. The gel was solidified by incubating at 37°C for 90 minutes, and then the well was flooded with EMEM and 5 mg/mL BSA. The cells were treated with Y27632 or control PBS. The gels were incubated at 37°C with 5% CO_2. The initial gel diameter was 15 mm. The medium was replaced every 24 hours. The extent of contraction was calculated by subtracting the diameter of the well at a given time point from the initial diameter (15 mm).

Cell Proliferation Assay

Proliferation of RPE was measured by 3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenylte-2H-tetrazolium bromide (MTT), using a commercially available kit (Nacalai Tesque). Cells were plated in RPE medium containing 10% FBS at a density of 1×10^4 cells/well in 96-well plates and allowed to adhere for 18 to 24 hours. Cultures were then washed once with PBS and fed with RPE medium, without or with Y27632 (10, 50, and 100 μM). The cells were incubated for another 72 hours and finally treated with 5 mg/mL MTT for 4 hours at 37°C. The MTT solution was aspirated, and the formazan crystals were dissolved in detergent reagent for 10 minutes. The relative cell number was determined based on the optical absorbance of the formazan at 570 nm, using a control wavelength of 655 nm measured in an automatic plate reader (Bio-Rad).

Animal Preparation

All animal experiments were conducted according to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experimental manipulations were performed on left eyes only. Thirty pigmented rabbits of either sex weighing 1.5 to 2.0 kg were included in the study. Before each procedure, the rabbits were anesthetized with intramuscular injections of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg). The pupils of the experimental eyes were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride, and the cornea was anesthetized with 0.4% oxybuprocaine hydrochloride eye drops. After performing a 0.2-mL anterior chamber paracentesis, 0.4 mL of pure perfluoropropane

TABLE 1. Trypan Blue Exclusion Test to Evaluate Cytotoxicity of Y27632 on Cultured RPE Cells

	Exp. 1	Exp. 2	Exp. 3	Mean ± SD
RPE (control)	90.8	91.7	95.8	92.8 ± 2.6
RPE + Y27632 (100 μM)	91.6	93.6	98	94.4 ± 3.3

The percent viable cells from three independent experiments is shown. There is no significant difference between the two groups ($P = 0.539$ by Student's t-test).

(C_3F_8) gas was injected into the vitreous cavity with a 30-gauge needle, to induce vitreous liquefaction.

Experimental PVR Animal Model

PVR was induced in the left eyes of 30 pigmented rabbits, as described previously with slight modifications.[38] Briefly, gas vitrectomy was performed by injecting 0.4 mL of C_3F_8 into the vitreous cavity 4 mm posterior to the corneal limbus after anesthesia was induced. Ten days later, 0.1 mL of RPE medium containing 1×10^5 of RPE cells was injected into the vitreous cavity together with 0.1 mL of platelet-rich plasma (PRP), with a 30-gauge needle. The sixth-passage RPE cells were used in this model.

In the treated group, the experimental eye of each rabbit ($n = 15$) was injected with 58.5 μg Y27632 dissolved in 0.1 mL physiological saline (Santen Pharmaceuticals, Osaka, Japan) immediately after RPE cell injection to achieve a final intraocular concentration of approximately 50 μM Y27632. For the control group ($n = 15$), 0.1 mL saline solution was injected. On days 7, 14, and 21, the treated rabbits continuously received the same volume of Y27632 injection; rabbits in the control group underwent a sham treatment. Each eye was examined by indirect ophthalmoscopy, and fundus video photographs were taken 3, 7, 14, 21, and 28 days after the RPE injection. The development of PVR was evaluated on videography in a masked fashion, and the PVR was graded according to the scale of Fastenberg et al.[39] The grading is as follows: 0, no abnormality; 1, vitreous strand; 2, traction of the retina; 3, partial retinal detachment (less than two quadrants); 4, extended (more than two quadrants) but not total retinal detachment; 5, total retinal detachment. A grade of 3 to 5 was defined as TRD.

Statistical Analysis

Student's t-test and the Fisher exact test were used for statistical analysis. $P < 0.05$ was considered to be statistically significant.

RESULTS

Toxicologic Experiment on Cultured RPE Cells

We used the trypan blue exclusion test to determine whether Y27632 has toxicologic effects on cultured RPE cells (Table 1). No differences in cell viability were found between cells exposed to Y27632 and those not exposed. The data suggest that 100 μM of Y27632 does not have any obvious cytotoxic effect on cultured RPE cells.

α-SMA Expression in RPE Cells

Increased contractile activity of cultured RPE during passage is thought to be closely related to transdifferentiation of RPE cells based on the morphologic change into myofibroblast-like cells, and increased expression of α-SMA.[7] First, we confirmed the expression of α-SMA that appears according to the transdifferentiation, in our RPE. In the third-passage RPE cells, most cells maintained the epithelial morphologic appearance with expression of α-SMA in only approximately 20% of cells (Fig. 1A). The sixth-passage RPE cells appeared more fibroblast-like, and

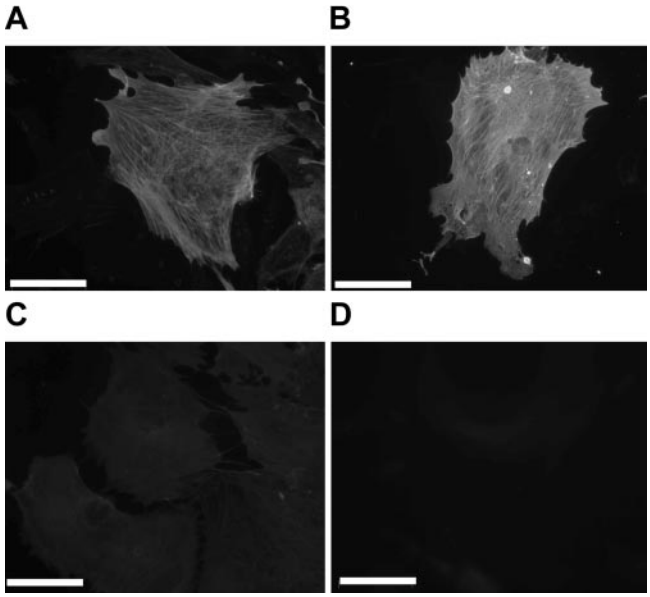

FIGURE 1. RPE cell phenotype changed as passaged. (**A**, **B**) Expression of α-SMA in the third- and sixth-passage RPE cells, respectively. The expression of α-SMA increased during passages. Control experiments for third- (**C**) and sixth-passage (**D**) RPE cells are provided to rule out nonspecific staining. Bar, 200 μm.

expression of α-SMA increased (Fig. 1B). Western blot analysis revealed increased expression of α-SMA in sixth-passage RPE cells compared with third-passage RPE cells (Figs. 2A, 2B). Quantitative analysis of three independent immunoblot results using densitometry confirmed a significantly higher α-SMA expression level in sixth-passage RPE cells ($P < 0.01$, Fig. 2C).

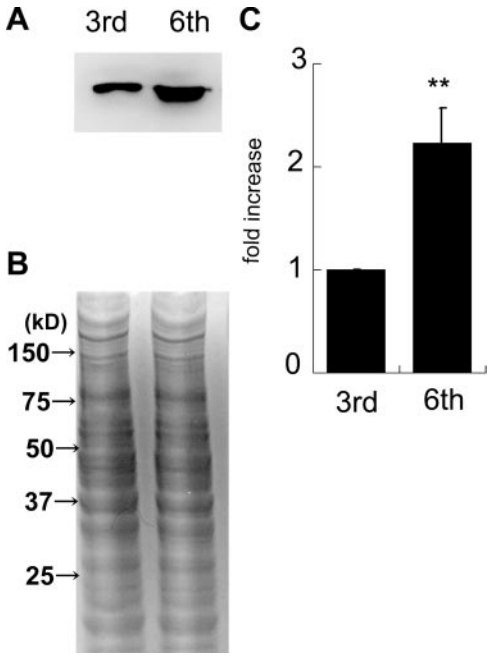

FIGURE 2. (**A**) Immunoblotting data reveal the increased expression of α-SMA in sixth-passage RPE cells and (**B**) Coomassie blue staining of the gel is provided as a lysate control. (**C**) Quantitative analysis of three independent experiments using gel analyzer showed an approximate 2.2-fold increase of α-SMA expression in sixth-passage compared with third-passage RPE. $^{**}P < 0.01$ by Student's t-test.

Effect of Y27632 on α-SMA Expression in Cultured RPE Cells

Because expression of α-SMA is essential for inducing force generation of myofibroblasts in vitro and in vivo,[17,40] we determined whether administration of Y27632 inhibits α-SMA expression in cultured RPE cells (Figs. 3, 4). RPE cells were cultured and passaged under normal conditions. Y27632 then was added at a final concentration of either 10 (Fig. 3) or 50 (Fig. 4) μM into the media, and the cells were cultured for 24 hours. Thereafter, the media were replaced with RPE media without the drug, and culture was continued for another 24 hours (recovery 24 hours). The cells were fixed at the time indicated, and immunostaining for α-SMA was performed. The study showed that α-SMA expression was strongly suppressed with either drug concentration (Figs. 3A, 4A). The data also showed that the expression recovered 24 hours after withdrawal of the drug (Figs. 3, 4).

Immunoblot analysis also revealed that Y27632 suppressed α-SMA expression in RPE cells in the presence of either 10 or 50 μM of Y27632 (Figs. 3B, 4B). Quantitative analysis using densitometry from three independent experiments showed that α-SMA expression level in RPE cells was significantly lower than in the control 24 hours after administration of 10 μM of Y27632 ($P < 0.05$, Fig. 3C). The higher concentration (50 μM) of the drug had a greater inhibitory effect, and the α-SMA expression level was significantly reduced at both 6 and 24 hours ($P < 0.05$ and $P < 0.01$, respectively, Fig. 4C). There was no significant difference between results in control experiments and the 24-hour recovery period at either concentration.

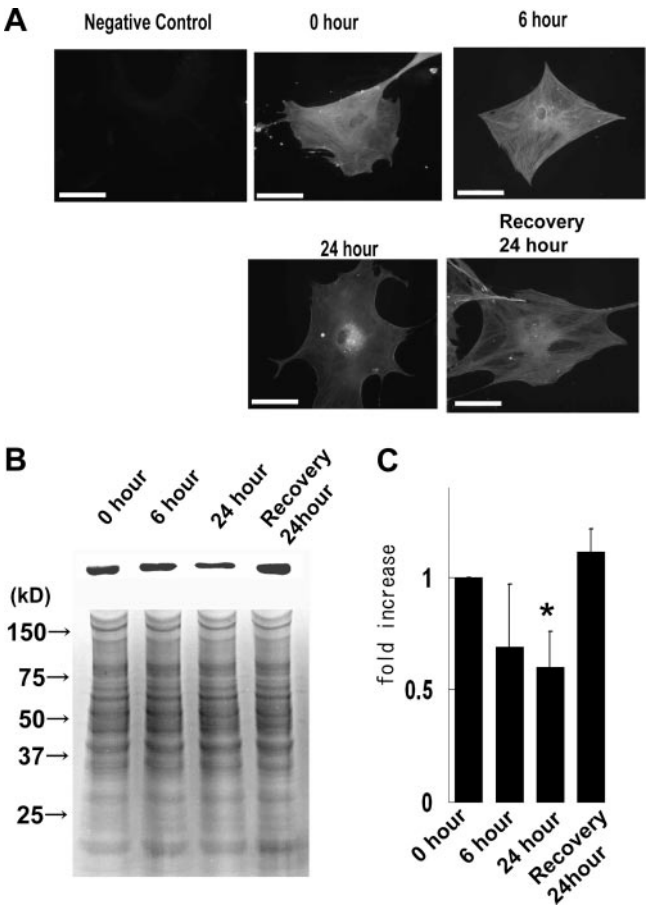

FIGURE 3. Sixth-passage RPE cells were treated with 10 μM Y27632, and the expression of α-SMA was studied by immunostaining (**A**) or immunoblot analysis (**B**) at the time indicated. The α-SMA decreased with Y27632 treatment and recovered after drug removal. Coomassie blue staining of the gel served as a lysate control that confirmed similar amounts of cell lysates were loaded in each lane in this experiment. (**C**) Quantitative analysis of three independent experiments showed that the α-SMA expression level was significantly reduced ($P < 0.05$) after 24 hours. The expression level during the 24-hour recovery period was similar to baseline, and there was no significant difference. Scale bars, 200 μm.

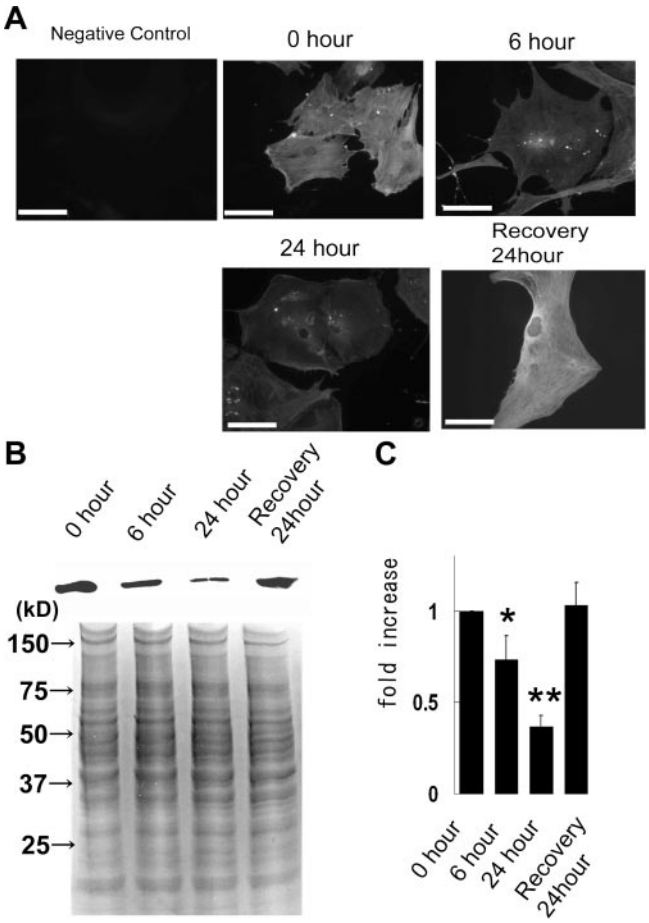

FIGURE 4. As in Figure 3, 50 μM Y27632 was tested to determine whether α-SMA expression is suppressed in the sixth-passage RPE cells. Immunostaining (**A**) or immunoblot results (**B**) show that 50 μM Y27632 inhibited α-SMA expression more than did 10 μM (Fig. 3). (**C**) Densitometric analysis of three independent immunoblot results shows that the α-SMA expression level was significantly reduced ($P < 0.05$ after 6 hours, and $P < 0.01$ after 24 hours). The expression level during the 24-hour recovery period was similar to baseline, and there was no significant difference. Scale bars, 200 μm.

Effect of Y27632 on Cell-Induced Collagen Gel Contraction and Cell Proliferation

Because the contractile activity of the RPE cells on the extracellular matrix in vivo is critical for the development of TRD, we investigated the effect of Y27632 on this biological event. The type I collagen gel contraction assay is one method that is widely used for cells in culture. We used the sixth-passage RPE cells in this assay. RPE cells were harvested in type I collagen gel, and the cells then were left unstimulated. Either a concentration of Y27632 or control PBS was added to the media, and gel contraction was measured 48 hours later. Y27632 significantly inhibited RPE-induced gel contraction in a dose-dependent manner (Table 2).

TABLE 2. Effect of Y27632 on Type I Collagen Gel Contraction in Sixth-Passage RPE Cells from Three Independent Replicates

	Exp. 1	Exp. 2	Exp. 3	Mean ± SD
RPE (control)	3	3.3	3.5	3.3 ± 0.25
RPE + Y27632 (10 μM)	2.8	2.4	1.9	2.4 ± 0.45*
RPE + Y27632 (50 μM)	1.2	1	0.6	0.9 ± 0.3**
RPE + Y27632 (100 μM)	0.1	0.1	0.2	0.1 ± 0.1**

Data indicate contraction after 48 hours (in millimeters) in three experiments.

* $P < 0.05$ and ** $P < 0.01$ by Student's *t*-test compared with the control.

The effect of the drug on cell proliferation was also tested. Rabbit RPE cells were harvested in 96-well plates, and an MTT assay was performed. Only the sixth-passage RPE cells were used. The number of cells was similar among the groups (Fig. 5), indicating that Y27632 does not significantly affect RPE cell proliferation.

Effect of Y27632 on PVR in a Rabbit Model

Figure 6 shows the percentage of TRD in the rabbits that received RPE and PRP, followed by either Y27632 or the control saline solution injection every week. The PVR score was lower in rabbits that received Y27632 than in control animals. On day 3, TRD had not developed in any eye in either the drug or the control group. On day 7, no treated eyes had TRD, whereas 3 (20%) of 15 control eyes showed TRD. On day 14, TRD developed in 2 (13%) of 15 treated eyes and in 10 (67%) of 15 control eyes. There was no significant difference between the two groups within the first 14 days. The difference in the rate of TRD development became more prominent in the later phase (days 21 and 28). Only 5 (33%) and 8 (53%) of 15 eyes had TRD in the treated group on days 21 and 28, respectively, whereas 13 (86%) and 14 (93%) of 15 control eyes, respectively, showed development of TRD at those time points ($P < 0.05$ at both time points).

DISCUSSION

The origin of α-SMA–positive myofibroblast cells in human PVR has not been clarified, some studies excluded RPE cells because of the absence of keratin staining.[10] Our data confirmed that after being passaged six times, dedifferentiated RPE cells lost their epithelial cell appearance and α-SMA expression

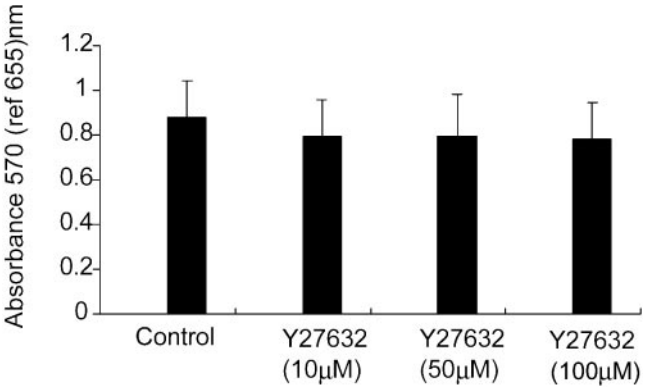

FIGURE 5. Sixth-passage RPE cells were harvested and incubated in 96-well dishes, with or without Y27632 and an MTT assay was used to evaluate the proliferative potential of the cells. The data are expressed as the mean ± SD of absorbance. Y27632 did not inhibit proliferation in any of the dose groups.

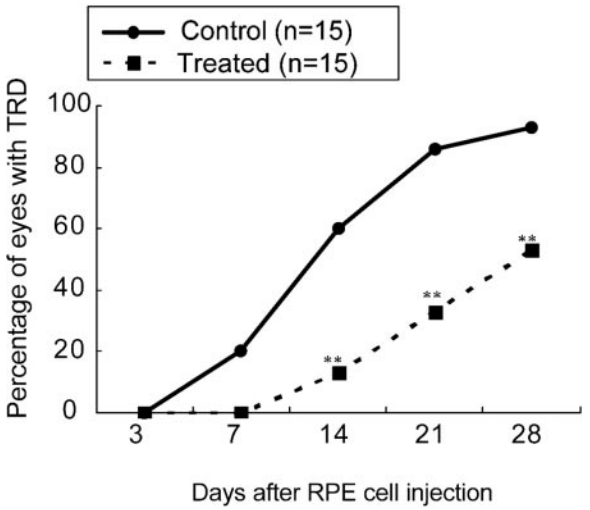

FIGURE 6. The percentage of TRD in the rabbits that received RPE and PRP, followed by either Y27632 or the control saline solution injection every week. On day 3, no eyes in either the drug or control group showed development of TRD. On day 7, no treated eyes showed TRD, whereas 3 (20%) of 15 control eyes showed TRD. There was no significant difference between the two groups within the first 14 days. On day 14, TRD developed in 2 (13%) of 15 treated eyes and in 10 (67%) of 15 control eyes. The difference in the TRD percentage became more significant in the late phase. On days 21 and 28, respectively, only 5 (33%) and 8 (53%) of 15 eyes in the treated group and 13 (86%) and 14 (93%) of 15 eyes in the control group showed TRD. **Statistically significant difference ($P < 0.01$) compared with control animals.

increased significantly. In addition, TGFβ is upregulated in PVR[41,42] and RPE cells in culture express α-SMA after passage or stimulation with TGFβ.[8,9] These findings suggest that increased amounts of TGFβ in the vitreous may lead to transdifferentiation of migrated RPE cells and consequent upregulation of α-SMA in PVR.

Although Y27632 attenuated progression of rabbit PVR, the mechanism is unknown. Other investigators have suggested the importance of cell-mediated contractile activity in rabbit PVR model based on the finding that fibroblasts most effective at inducing PVR display the most potent activity in the in vitro contraction assay.[38] The present study showed that contractile activity of the RPE cells was suppressed by Y27632 in type I collagen gel in vitro, which is known as one of the main components of ERM.[43] Taken together, these findings show that Y27632 may attenuate development of PVR by suppressing contraction of ERM by RPE cells in this model. Thus, a future approach to prevention of PVR could target the contractile activity of RPE cells in the vitreous.

It has been shown that increased α-SMA expression is sufficient to enhance fibroblast contractile activity, and administration of fusion peptide of α-SMA inhibits contractile force generation in vitro and in vivo.[17,40] Taken together, these and the current findings show that Y27632 suppresses type I collagen gel contraction in RPE cells, probably by suppressing expression of α-SMA, which may lead to attenuation of PVR. Y27632 did not inhibit RPE proliferation in vitro. This is consistent with the finding that irradiated cells could still induce PVR in rabbits,[39] indicating that proliferative potential is not critical for inducing PVR in an animal model.

Another possible mechanism by which Y27632 suppresses development of PVR is by inhibiting synthesis of type I collagen. Some investigators have reported that Y27632 reduces the type I collagen mRNA level in a model of rat liver fibrosis or in cultured hepatic stellate cells.[30,44] Although these findings are

not commonly observed among cells including RPE and retinal glial cells, Y27632 may have attenuated PVR by suppressing synthesis of type I collagen by corresponding cells. Further investigation is necessary to understand how Y27632 affects attenuation of PVR.

The effects of Y27632 were reversible, which indicates that they were not the result of cellular toxicity or apoptosis. This finding is consistent with the in vitro finding in trypan blue exclusion test in this study (Table 1). They indicate the relative safety of Y27632. However, longer observations may be needed in vivo and in vitro to evaluate drug toxicity precisely.

Growth factors are involved in PVR, including platelet-derived growth factor (PDGF) and TGFβ, and inhibiting these factors can be a prophylactic treatment.[45–47] However, it may be difficult to prevent disease progression completely by blocking one specific factor. An inhibitor of a downstream effector, such as Rho-kinase inhibitor, could be more useful because the effect is broader. For instance, the RPE reportedly has an autocrine loop of growth factors such as PDGF, TGFβ, and interleukin-10, all of which contribute partly to the contraction.[48] However, the present study showed complete inhibition of the contractile activity in vitro at the highest concentration.

The combined results of the present in vitro and in vivo experiments demonstrated that Y27632 inhibited RPE α-SMA stress fiber formation and then suppressed the contraction of ERM in a rabbit PVR model. These findings also suggest that inhibition of the Rho-P160ROCK pathway may be a new strategy for combating PVR fibrosis.

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