Epithelial-Mesenchymal Transition-Like Phenotypic Changes of Retinal Pigment Epithelium Induced by TGF-β Are Prevented by PPAR-γ Agonists

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Purpose. Proliferative eye diseases, such as proliferative vitreoretinopathy and proliferative diabetic retinopathy, are caused partly by fibrotic change of retinal pigment epithelial cells (RPECs). The purpose of our study was to examine the effect of the peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist on the fibrotic change of primate RPECs.

Methods. Monkey RPECs (MRPECs) isolated from a cynomolgus monkey eye were subcultured. To induce fibrotic change, MRPECs were cultured with TGF-β2 (3 ng/mL), and also cultured in the coexistence of TGF-β2 and the PPAR-γ agonist pioglitazone (30 μM). The phenotype of the cultured MRPECs was evaluated by phase contrast microscopy and immunocytochemical analysis. The phosphorylation of Smad2/Smad3 proteins was examined by Western blot analysis.

Results. Primary MRPECs were cultured as a monolayer with a hexagonal cell shape, and positive expression of ZO-1, Na^+/K^+-ATPase, and RPE65 was confirmed. Cell morphology and the expression of these markers were maintained in the presence of pioglitazone, whereas the cells were elongated and the expression of these markers was reduced in its absence. Conversely, the expression of phalloidin, α-smooth muscle actin, and fibronectin was reduced in the presence of pioglitazone, whereas it was increased in the absence. Western blot assay demonstrated that phosphorylation of Smad2/Smad3 proteins was suppressed by pioglitazone.

Conclusions. The PPAR-γ agonist pioglitazone inhibited the fibrotic change of primary MRPECs through the suppression of TGF-β signaling. Pioglitazone might prove to be a clinically applicable and effective pharmaceutic treatment for proliferative eye diseases. (*Invest Ophthalmol Vis Sci.* 2012;53:6955–6963) DOI:10.1167/iovs.12-10488

Intraocular proliferative diseases, such as proliferative vitreoretinopathy (PVR) and proliferative diabetic retinopathy (PDR), are the major cause of vision loss and have poor visual prognosis in spite of the development of innovative surgical techniques and anti-VEGF agents.[1,2] Despite the vigorous accumulation of knowledge about the pathology of PVR over the past decade, little progress has been made toward the clinical management of the disease.[2] To date, to our knowledge no practical pharmacologic treatment has been developed to repair the damaged fibrotic tissues involved in these diseases.

Intraocular fibrosis is a clinically-recognized, underlying pathologic feature in PVR and PDR that leads to functional impairment of the retina. The fibrotic change involves sub- and preretinal fibrosis, scarring, and proliferative membrane. These fibrotic features result in the recurrence of the disease and aggravate the prognosis of visual acuity.[3,4] Such tissue fibrosis also is found in a variety of tissues, such as those of the kidney, liver, lung, and so forth. The fibrous tissue reduces the flexibility of the detached retina and becomes a major cause for failure of retinal reattachment surgery. Once fibrosis occurs, a surgical operation thus far has been the only possible therapeutic modality. In the pathogenesis of PVR and PDR, changes such as proliferation and production of the fibrillar extracellular matrix (ECM) on the retina, occur frequently in retinal pigment epithelial cells (RPECs) located in the vitreous cavity and subretinal space.[2,5] Thus, agents capable of preventing the fibrotic change of RPECs may be of great therapeutic value in retinal reattachment surgery. Numerous drugs have been tested on animal models or cell cultures to inhibit cell proliferation and proliferative membrane formation. However, many of these drugs cause severe side effects and only a few have been used in clinical trials.[2,6]

The epithelial mesenchymal transition (EMT) or transdifferentiation of epithelial cells has been theorized to have a critical role in the development of such pathologic fibrosis.[7–10] In fact, TGF-β signaling has been shown to have a crucial role in these fibrotic changes.[11,12] Recently, numerous reports have demonstrated that treatment with a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist attenuates experimentally-induced kidney,[13,14] liver,[15] lung,[16] skin,[17] and cardiac fibrosis.[18] In a variety of renal diseases, overexpression of TGF-β isoforms is observed in animals and humans.[19–22] These reports also showed that the PPAR-γ agonist inhibited the fibrotic change by down-regulating the TGF-β pathway.

Likewise, the overexpression of TGF-β has been observed in the vitreous body in PDR and PVR,[23,24] and also has been investigated in relation to the proliferative membranes in these diseases.[25] The purpose of our study was to investigate if a human RPE cell line and primate RPECs exhibited fibrotic changes by TGF-β2, and if PPAR-γ agonists could prevent this fibrotic process. Our findings demonstrated that

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the PPAR-γ agonist restored the fibrotic pathologic changes mediated by TGF-β2.

Methods

Materials

For our study, recombinant human TGF-β2 was purchased from R&D Systems, Inc. (Minneapolis, MN). Pioglitazone was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). Dulbecco's modified Eagle medium:Nutrient Mixture (DMEM/F12), penicillin, streptomycin, Alexa Fluor 546 phalloidin, Alexa Fluor 488 donkey anti-mouse IgG, and Alexa Fluor 594 donkey anti-rabbit IgG were purchased from Life Technologies Corporation (Carlsbad, CA). Dispase II was purchased from Roche Applied Science (Penzberg, Germany), and FNC Coating Mix was purchased from Athena Environmental Sciences, Inc. (Baltimore, MD). ZO-1 polyclonal antibody was purchased from Zymed Laboratories, Inc. (South San Francisco, CA), Na^+-K^+-ATPase monoclonal antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and DAPI was purchased from Vector Laboratories, Inc. (Burlingame, CA). N-cadherin and fibronectin antibody were purchased from BD Biosciences Pharmingen (Franklin Lakes, NJ). Smad-2, Smad3, and phosphorylated Smad2 (phospho-Smad2) and phosphor-Smad3 antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Alpha smooth muscle (αSMA) antibody was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). RPE65 antibody and GAPDH were purchased from Abcam, Inc. (Cambridge, MA). Phosphatase inhibitor was purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Animal Experiment Approval

In all experiments, animals were housed and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Culture and Treatment of ARPE-19

ARPE-19, a human RPE cell line, was purchased from the American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin in a humidified atmosphere at 37°C in 5% CO_2 for more expansion.[26] As described previously, the cells were treated in the fresh medium with recombinant human TGF-β2 (5 ng/mL, fibrotic group) or serum-free medium (control group) after 24 hours of serum starvation when the cells reached confluency.[27] Next, we investigated if a difference could be found between the control and fibrotic groups in the morphology of ARPE-19. The ratio of elongated cells cultured under various conditions then was calculated. The elongated cells were defined as ones in which the length of the cell is two times longer than the width of that cell. ARPE-19 cells were seeded at a density of 1.0×10^5 cells onto 12-well plates in the medium containing 10% FBS. PPAR-γ agonist was added at the concentration of 10 and 30 μM with TGF-β2 (PPAR-γ group) simultaneously.

Cell Culture and Treatment of Monkey RPECs (MRPECs)

MRPECs were cultured from the posterior area of an eyeball enucleated from a cynomolgus monkey (3–5 years old, estimated equivalent human age 5–20 years) housed at Nissei Bilis Co., Ltd. (Otsu, Japan). The MRPECs then were separated from the RPEC fragments in accordance with the method described previously for human fetal RPE.[28] The MRPECs then were cultured on FNC Coating Mix-coated dishes in DMEM/F12 supplemented with 10% FBS, 50 U/mL of penicillin, and 50 μg/mL of streptomycin for more expansion in a humidified atmosphere at 37°C in 5% CO_2. The culture medium then was changed every 2 days. When the cells reached confluency in 5 to 7 days, they were rinsed in Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS), trypsinized with 0.05% Trypsin-EDTA (Life Technologies) for 5 minutes at 37°C, and passaged at ratios of 1:2 to 4. Cultivated MRPECs at passages 1 to 3 were used for all experiments. MRPECs then were treated with recombinant human TGF-β2 (3 ng/mL) (fibrotic group) or 2% FBS-containing medium (control) after 24 hours of serum starvation when the cells reached confluency. As a PPAR-γ group, MRPECs were cultured with medium containing TGF-β2 and PPAR-γ agonist (30 μM). The morphologic differences among the groups then were observed. The MRPECs then were seeded at a density of 1.0×10^5 cells onto 24-well plates in the medium containing 10% FBS to investigate if a difference in cell morphology could be found among the groups.

Immunocytochemistry

To analyze the expression and localization of function-related proteins in the cells, ARPE-19 cells and MRPECs were cultured at a density of 3×10^4 cells/cm^2 on Lab-Tek Chamber Slides (NUNC A/S, Roskilde, Denmark) in various conditions for 48 hours before staining. The cells then were stained with individual antibodies; namely, anti-ZO-1, anti-Na^+/K^+-ATPase, anti-RPE65, anti-N cadherin, anti-E cadherin, anti-phalloidin, anti-fibronectin, and anti-α smooth muscle antibodies. For a second antibody, 1:2000 diluted Alexa Fluor 488 donkey anti-mouse IgG and 1:2000 diluted Alexa Fluor 594 donkey anti-rabbit IgG were used. Cell nuclei then were stained with DAPI, and the slides were inspected by use of a fluorescence microscope.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For RT-PCR, total RNA was extracted by use of TRIzol reagent (Life Technologies) and treated with RNase-free DNase I (Roche). cDNA was synthesized with SuperScript III Reverse Transcriptase (Life Technologies) and PCR reactions then were performed with EX Taq DNA polymerase (Takara Bio, Shiga, Japen) as follows: denaturation at 94°C for 30 seconds, 23 to 35 cycles of annealing at 55°C to 57°C for 30 seconds, and elongation at 72°C for 30 seconds. The PCR products then were separated by electrophoresis on 2% agarose gels and detected under ultraviolet illumination.

Real-Time Quantitative PCR (qPCR)

For the real-time qPCR, reverse transcription was conducted using the TaqMan Fast Advanced Master Mix (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's protocol. Real-time qPCR reactions then were conducted by use of the StepOnePlus (Applied Biosystems) real-time PCR system according to the manufacturer's protocol.

Western Blot Analysis

For the detection of Smad2, Smad3, phospho-Smad2, and phospho-Smad3, MRPECs were serum-starved for 24 hours and then incubated with TGF-β2, or TGF-β2 and PPAR-γ agonist-containing medium for 10 minutes and extracted with Tris-buffered saline containing phosphatase inhibitors. The proteins then were separated by use of SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The blots were blocked overnight with nonfat dried milk and incubated with rabbit anti-human phospho-Smad2 monoclonal antibody, rabbit anti-human phospho-Smad3 monoclonal antibody, rabbit anti-human Smad2, or rabbit anti-human Smad3. Luminescence was observed by use of an ImageQuant LAS-4000

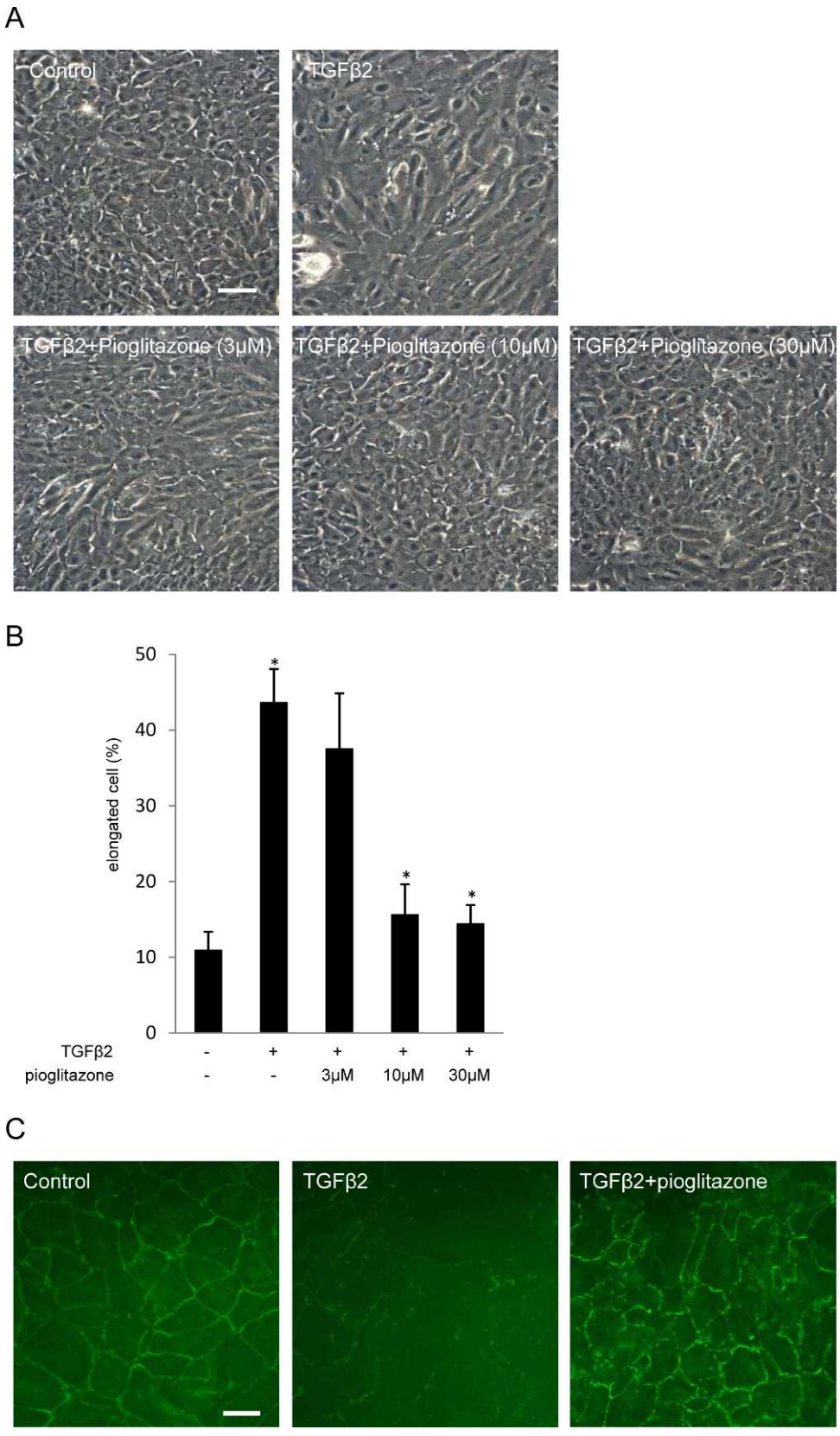

Figure 1. Fibrotic change induced by TGF-β2 and the inhibitory effect of pioglitazone on the morphologic change in ARPE-19 cells. Phase-contrast light microscope images of ARPE-19 cells with TGF-β2 (5 ng/mL) and pioglitazone (3, 10, 30 μM; [**A**]), the ratio of elongated cells in each condition (i.e., the control, TGFβ and PPAR-γ conditions; [**B**]; $n = 4$; $P < 0.005$), and immunocytochemical staining of ZO-1 (**C**) are shown. *Scale bar*: 100 μm.

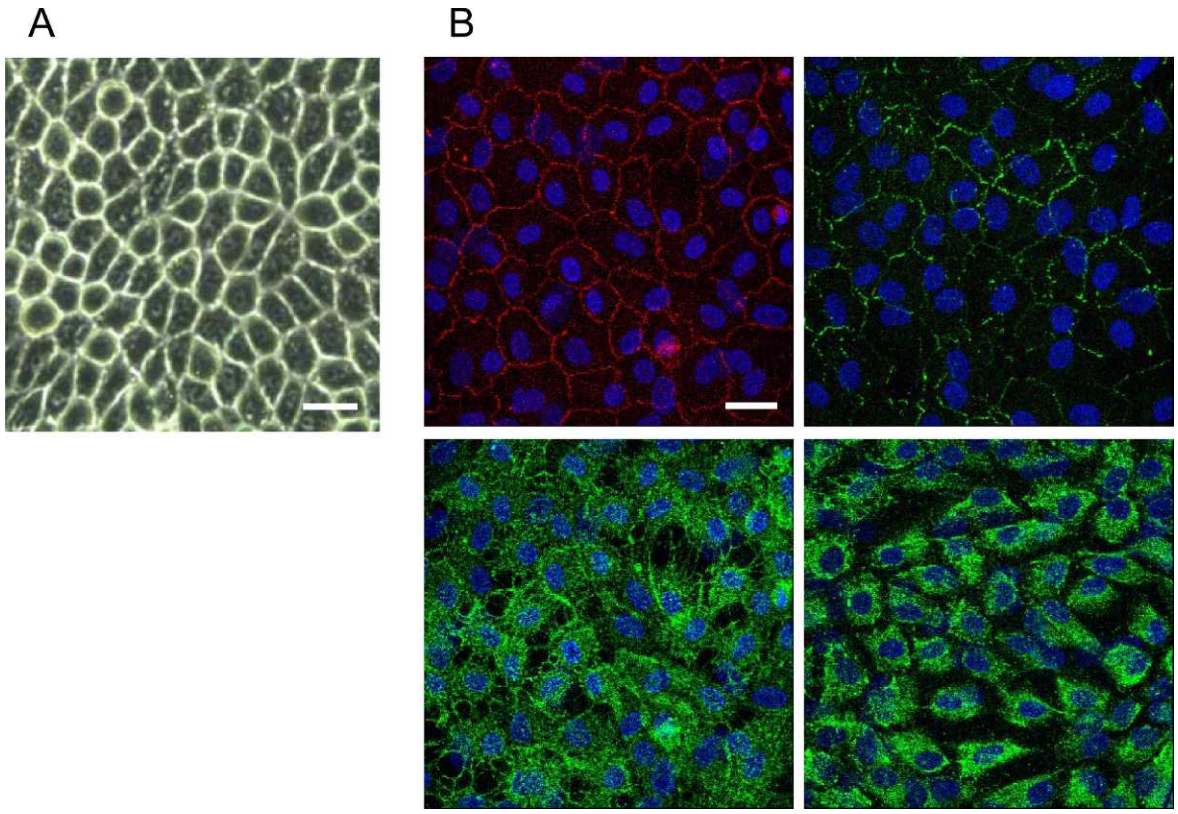

Figure 2. Images of primary MRPECs ($P = 1$) immunocytochemically stained (**A**) with ZO-1 (*top left*), N-cadherin (*top right*, *green*), Na^+/K^+-ATPase (*bottom left*), RPE65 (*bottom right*, *green*), and a phase-contrast light microscopy image (**B**). *Scale bar*: 100 μm.

mini (FUJIFILM, Tokyo, Japan) dedicated charge-coupled device (CCD) camera system.[29]

Results

Preventive Effect of PPAR-γ Agonist against the TGF-β2–Induced Morphologic Change in ARPE-19

The cellular morphology of ARPE-19 cells is the characteristic cuboidal shape under normal culture conditions. On the other hand, the presence of TGF-β2 (5 ng/mL) induced an EMT-like morphologic change of ARPE-19 cells, symbolized by the elongated and fibroblastic phenotypes (Fig. 1A). TGF-β2 at 5 ng/mL, a concentration that is nontoxic and is known to be the most effective, was used in the following experiments. When cells were treated simultaneously with TGF-β2 and PPAR-γ agonist (pioglitazone), pioglitazone at 3 μM demonstrated a mixture of cuboidal and elongated cell shapes, while pioglitazone at 10 and 30 μM reversed the fibroblastic cell shape mediated by TGF-β2 to the cuboidal cell shape. Those results indicated that the PPAR-γ agonist has a concentration-dependent preventive effect against the fibrotic or EMT-like phenotypic change of ARPE-19 cells induced by TGF-β2 (Fig. 1B). The preventive effect of pioglitazone toward the TGF-β2-mediated phenotypic changes of ARPE-19 was confirmed further by use of ZO-1, one of the major epithelial functional tight junction molecules. The immunostaining of ARPE-19 cells with ZO-1 antibody showed the characteristic staining pattern of ZO-1 at the plasma membrane, while cells treated with TGF-β2 greatly reduced the ZO-1 staining potential. On the other hand, cells treated with TGF-β2 and pioglitazone demonstrated the cuboidal cell shape with the distinct ZO-1 staining pattern (Fig. 1C).

Influence of PPAR-γ Agonist and TGF-β2 on the Expression of Functional Molecules in MRPECs

The established MRPEC cultures were maintained in DMEM/F12 medium containing 2% FBS, a concentration that minimizes the TGF-β activity of FBS, and the MRPECs demonstrated the characteristic hexagonal or polygonal morphology (Fig. 2A). That culture condition also maintained the expression of functional proteins, such as ZO-1, Na^+/K^+-ATPase, N-cadherin, and RPE65 (an essential protein for the visual cycle), and the proteins were expressed according to their subcellular localization (Fig. 2B).

When MRPECs were treated with TGF-β2, they lost their characteristic polygonal cell morphology. However, simultaneous treatment of the cells with TGF-β2 and pioglitazone reversed the cell shape change induced by TGF-β2 (Fig. 3A). Those results indicated the induction of the EMT-like or fibrotic change of MRPECs by TGF-β2 (Fig. 3A). TGF-β2 (3 ng/mL) was chosen as the optimal concentration for the induction of the fibrotic change of the MRPECs. The morphologic changes of the MRPECs induced by TGF-β2 (the EMT-like or fibrotic change) were reduced in the presence of pioglitazone below those in the TGF-β2 group.

The changes of distribution of ZO-1 (Fig. 3B), Na^+/K^+-ATPase (Fig. 3C), and N-cadherin (Fig. 3D) in the MRPECs were observed in the TGF-β2–treated group when compared to those of control cells. However, the TGF-β2 changes were inhibited by the PPAR-γ agonist pioglitazone (Figs. 3B, 3C, 3D). The expression of RPE65 and N-cadherin was examined further by use of real-time qPCR, which showed that the RPE65 expression was reduced markedly by TGF-β2, while the N-cadherin mRNA was increased by TGF-β2. Of interest, pioglitazone blocked such TGF-β2–mediated changes (Fig. 3E). The same tendency was observed in N-cadherin mRNA expressions using real-time PCR (Fig. 3F).

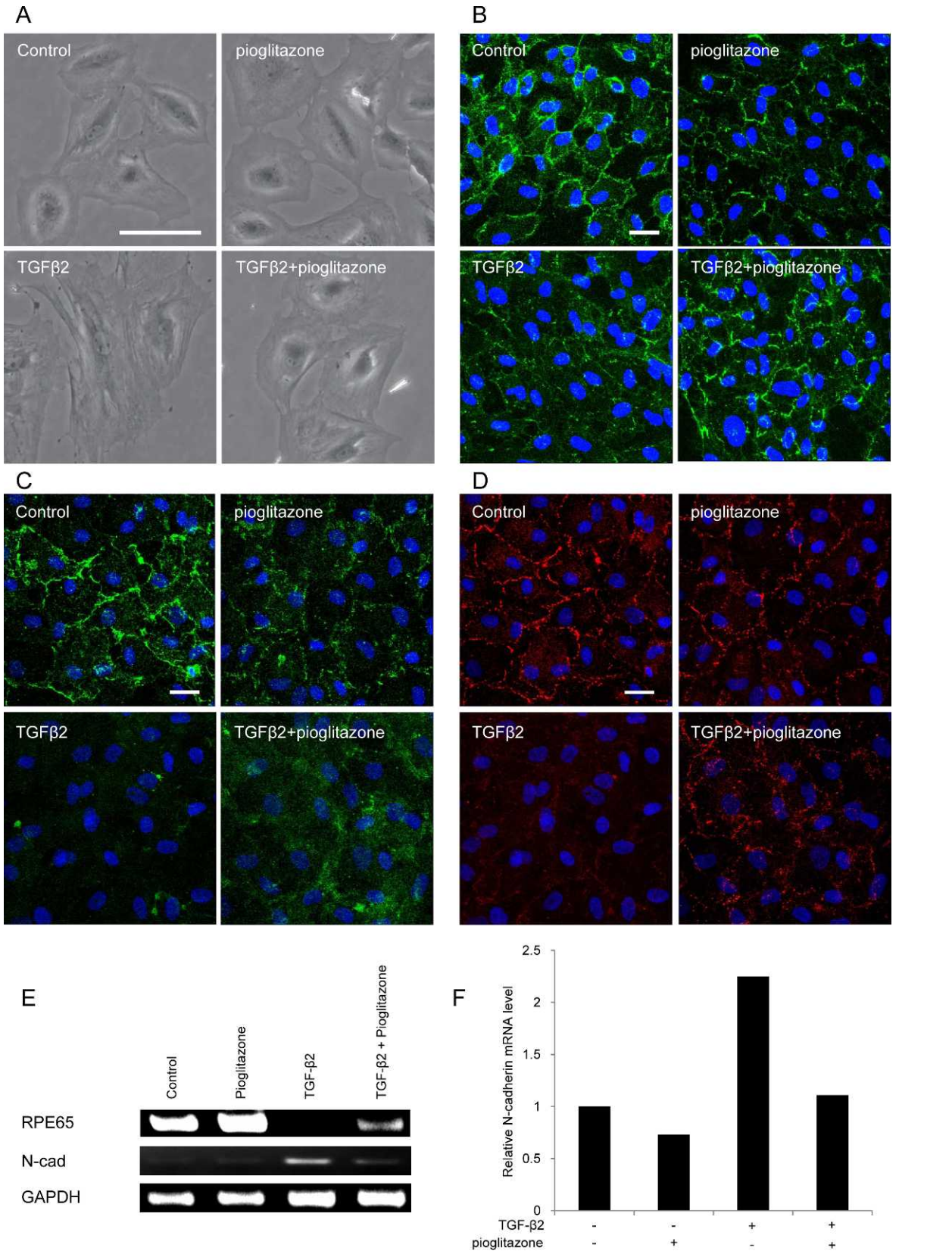

Figure 3. The effect of pioglitazone on fibrotic change induced by TGF-β2 in primary MRPECs. Phase-contrast light microscopy images of primary MRPECs (**A**), and the change of distribution in the expression of N-cadherin (**B**), Na^+/K^+-ATPase (**C**), and ZO-1 (**D**) in the medium with TGF-β2 (3 ng/mL) and/or pioglitazone (30 μM). (**E**) An image showing the mRNA expression of RPE65 and N-cadherin. (**F**) An image showing the ratio of the expression of N-cadherin mRNA as compared to the control. *Scale bar*: 100 μm.

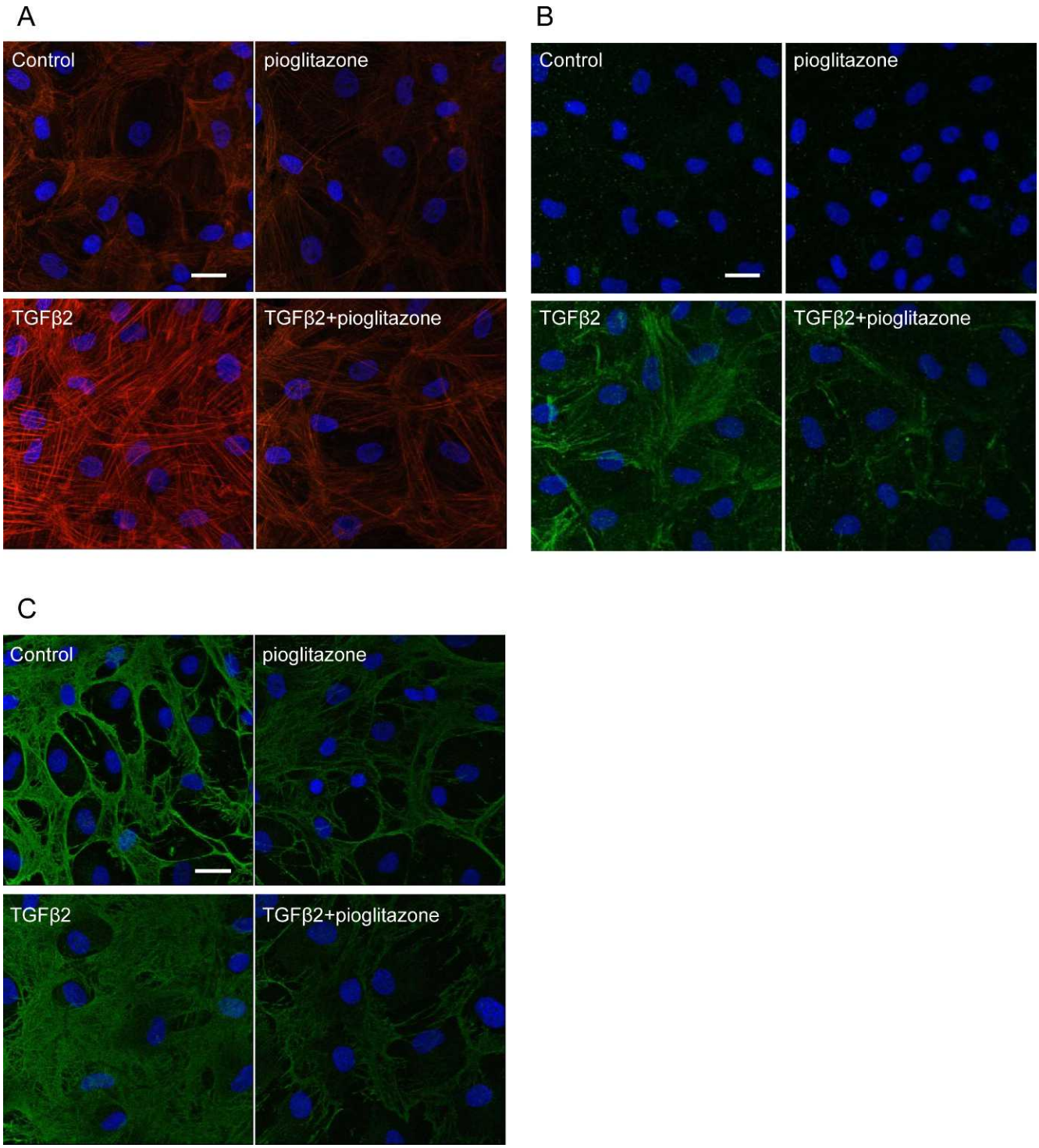

Figure 4. The effect of pioglitazone and TGF-β2 on the cytoskeleton of MRPECs. Images showing the immunostaining of phalloidin (**A**), αSMA (**B**), and fibronectin (**C**). *Scale bar*: 100 μm.

Next, the expression of fibrotic or EMT-related markers, such as stress fibers (Fig. 4A), α-smooth muscle actin (α-SMA, Fig. 4B), and fibronectin (Fig. 4C) was determined. The control cells demonstrated a low level of these EMT-related markers. However, the expression of these proteins was increased by exposure to TGF-β2 and that increase was reversed almost to normal control levels by the addition of PPAR-γ agonist pioglitazone.

Effect of PPAR-γ Agonist and TGF-β2 on the Smad Pathway in MRPECs

Finally, we investigated if the Smad pathway was related to the aforementioned findings. The expression of phospho-Smad2 and phospho-Smad3 proteins was detected only faintly in the control cells (Figs. 5A, 5B); however, phosphorylation of

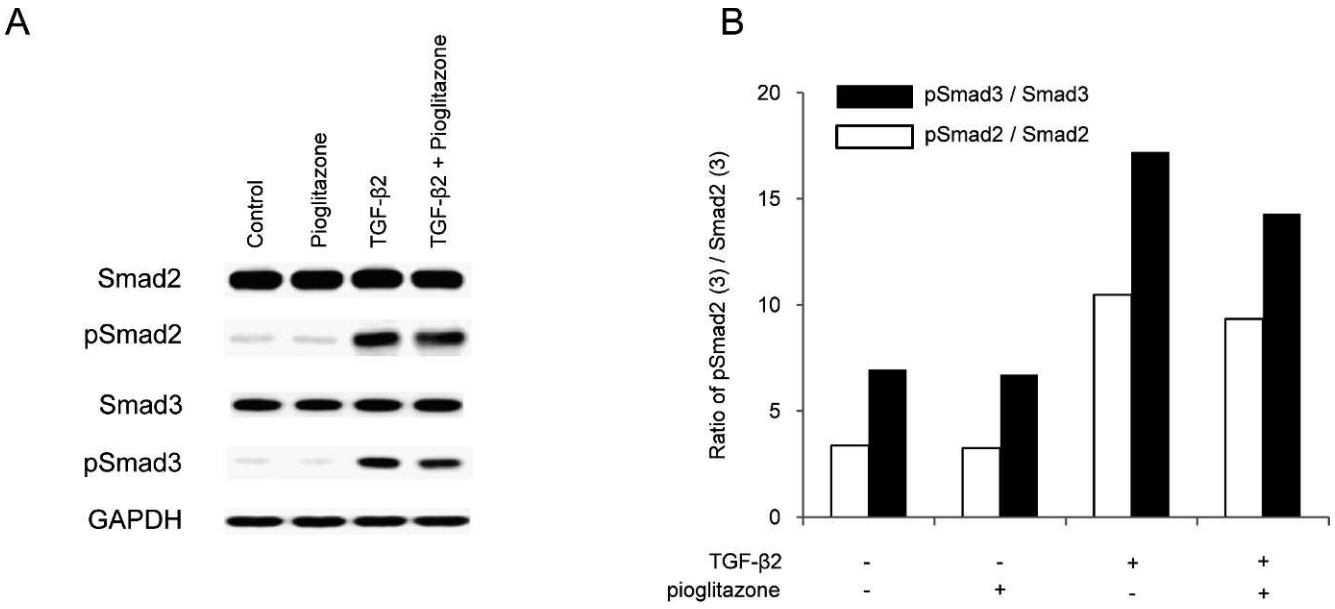

Figure 5. (**A**) The expressions of Smad2, phospho-Smad2 (pSmad2), Smad3, and pSmad3 after exposure to TGF-β2 and/or pioglitazone for 10 minutes. (**B**) The representative result is shown. Protein accumulation was assessed by densitometry ($n = 3$).

Smad2 and Smad3 was increased greatly in response to TGF-β2 stimulation (Figs. 5A, 5B). The PPAR-γ agonist tended to inhibit the phosphorylation of Smad2 and Smad3, yet at a low level (Fig. 5B).

Discussion

Intraocular fibrosis within a proliferative membrane is a clinically recognized underlying pathologic feature in PVR and PDR that leads to functional impairment of the retina. The proliferative membrane reportedly contains RPECs, hyalocytes, glial cells, and macrophage-derived cells,[30] and RPECs are known to have a key role in developing pathology. Numerous studies have reported the fibrotic- or EMT-like change by use of the ARPE-19 cell line, a human RPE cell line. Nonetheless, the established cell lines may have an intrinsic weakness, such as obtaining an abnormal chromosome along with passages. Thus, test results obtained through the use of these cells might not precisely mimic those of human primary cells. This concern led us to establish primate primary RPECs, similar to human primary RPECs.

TGF-β2[31] and vitreous specimens obtained from PVR or PDR[32] have been used to induce fibrotic change in ARPR-19 cells. The increased concentration of TGF-β2 in intraocular proliferative diseases is well known,[23] thus prompting us to investigate TGF-β2 as an inducer of fibrotic phase transition in our primate primary RPEC model. Fibrotic change of the primary MRPECs was observed as the accumulation of actin filaments in the cytoplasm. Overexpression of αSMA and fibronectin was detected, suggesting that TGF-β2 induces EMT-like phenotypic changes in the primary MRPECs similar to that in the human RPE cell line. These ECM proteins, major components of the proliferative membrane, are the typical pathologic features evident in intraocular proliferative diseases. Furthermore, expression of functional proteins, such as ZO-1, Na^+/K^+-ATPase, N-cadherin, and RPE65, was diminished at the cell surface in response to TGF-β2 stimulation. These findings suggested that we successfully made an in vitro model of an intraocular proliferative disease using primary MRPECs, and that RPECs lost the characteristic epithelial phenotypes and assumed EMT-like phenotypic changes by TGF-β2.

We further investigated the preventive effect of pioglitazone on the fibrotic- or EMT-like change induced by TGF-β2. A recent study showed that troglitazone, one of the PPAR-γ agonists, can prevent TGF-β2–induced EMT-like changes in the human RPE cell line.[33] Our results with pioglitazone, another PPAR-γ agonist, are consistent with the observation obtained from the human RPE cell line treated with troglitazone. The inhibitory effect of pioglitazone on EMT reportedly also has been observed in corneal keratocytes,[34] kidney cells,[14] and lung cells.[16] Importantly, our findings demonstrated the preventive effect of the PPAR-γ agonist pioglitazone on EMT by use of primary MRPECs. In this investigation, it is not uncovered if the effects of pioglitazone on the TGF-β2–induced EMT in cells are attributable to the PPAR-γ receptor-involved mechanism. Some of the glitazone members exhibit anti–TGF-β effects through non–PPAR-γ signaling. Thus, it is necessary to test whether or not the action reported here is via PPAR-γ. We currently are planning a future study to elucidate the molecular mechanism of the pioglitazone-mediated anti-EMT phenomenon in RPE cells, for example by using a dominant negative PPAR-γ system. The aim of our present study was to clarify the effect of PPAR-γ on the cynomolgus monkey primary RPE cells, instead of the long-term maintained cell lines. For the PPAR-γ overexpression system, it usually is necessary to use stable cell lines to investigate the intracellular events.

EMT can be induced or regulated by various growth and differentiation factors, including TGF-β, fibroblast growth factor, hepatic growth factor, and Wnt and Notch proteins.[35] Intracellular signaling molecules, such as p38 MAP kinase,[36] Notch,[37] Wnt,[38] NF-κB,[39] and phosphatidylinositol-3-OH kinase,[40] also reportedly are involved in the TGF-β signaling pathway. In the Smad pathway, after TGF-β binds to the receptor, the complex of phosphorylated Smad2/3 and Smad4 is translocated into the cell nucleus following gene overexpression in genes, such as *COL1A1*.[41] Of interest, our findings demonstrated that pioglitazone hampers phosphorylation of Smad2/3 activated by TGF-β2 in primate primary RPECs, yet at a low level. However, the precise mechanism by which pioglitazone induces the suppression of EMT in primate primary RPECs has yet to be elucidated.

The findings of our present study demonstrated that pioglitazone, a drug now being used for the treatment of diabetes mellitus, may hold the potential of being a clinically applicable pharmaceutic agent for the prevention or inhibition of intraocular proliferative diseases in the early stage of the pathology or if applied following surgery for retinal detachment. Further investigation using PVR, PDR, and AMD in vivo models is crucial to elucidate the pathology of these diseases and to discover clinically applicable therapeutic interventions for these diseases. Such future investigations hopefully will lead to the development of new drugs, such as pioglitazone.

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