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Suppression of Epithelial-Mesenchymal Transition by Prostaglandin E_2 in Retinal Pigment Epithelial Cells

Research Article

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Abstract

Background/Aims: An epithelial to mesenchymal transition of retinal pigment epithelium (RPE) cells plays an important role in the formation of proliferative fibrocellular membranes in individuals with proliferative retinopathy. Prostaglandin E_2 (PGE_2) exhibits various physiological actions including both pro- and anti-inflammatory effects. We have now investigated the effect of PGE_2 on the epithelial-mesenchymal transition (EMT) of mouse RPE cells and fibrotic reactions in a type I collagen gel.

Methods: Mouse RPE cells were incubated in three dimentional type 1 collagen gel culture system with transforming growth factor (TGF)-β2 (1ng/ml) in the absence or presence of PGE_2 at various concentration. The expression of α-smooth muscle actin as well as matrix metalloproteinase (MMP)-3, the phosphorylation of Smad2, paxillin were examined by immunoblot analysis. The release of MMP-2 and MMP-9 was examined by gelatin zymography.

Results: PGE_2 inhibited the TGF-β2–induced contraction of collagen gels mediated by RPE cells in concentration dependent manner. The expression of α–smooth muscle actin and fibronectin as mesenchymal markers was inhibited. The release of MMP–2 and MMP-9 as well as the phosphorylation of Smad2 and paxillin induced by TGF-β2 in RPE cells were also attenuated by PGE_2.

Conclusion: PGE_2 may inhibit the contraction of proliferative fibrocellular membranes that can lead to retinal detachment in individuals with progressive vitreoretinal diseases.

Keywords: Retinal Pigment Epithelial Cell; TGF-B2; Epithelial-Mesenchymal Transition; Collagen Gel Contraction; Prostaglandin E_2.

Introduction

Proliferative ocular diseases such as proliferative vitreoretinopathy (PVR) and proliferative diabetic retinopathy (PDR) can result in visual impairment or blindness. The contraction of proliferative tissue that has formed on or under the retina gives rise to retinal tearing and detachment and may be responsible for failure of surgery to repair retinal damage. The proliferative tissue consists of retinal pigment epithelium (RPE) cells, glial cells, and fibroblasts and is exposed to growth factors and inflammatory cytokines such as platelet-derived growth factor (PDGF), transforming growth factor–β (TGF-β), and hepatocyte growth factor (HGF). In particular, RPE cells that have undergone the epithelial-mesenchymal transition (EMT) play a key role in the formation of proliferative fibrocellular membranes [1]. These cells express α–smooth muscle actin (α-SMA), fibronectin, and vimentin, all of which are markers of the EMT, as well as matrix metalloproteinases (MMPs) and connective tissue growth factor (CTGF) [2]. Signaling by TGF-β and Smad proteins regulates the EMT, with the phosphorylation of Smad2 and Smad3 induced by TGF-β leading to the transcriptional activation of genes related to this phenotypic change. Mechanical forces generated by mesenchymal cells are required for the assembly of focal adhesion proteins such as focal adhesion kinase, paxillin, and vinculin.

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Prostaglandin E_2 (PGE_2) is produced widely throughout the body and has various biological effects. In particular, it plays a central role in the regulation of inflammation. PGE_2 exerts its effects through binding to E-prostanoid (EP) receptors, of which there are four subtypes (EP1, EP2, EP3, and EP4) [3]. These four subtypes of EP receptor are all members of the G protein–coupled receptor family, but they differ in the intracellular signaling pathways to which they are linked [4]. Most clinically beneficial effects of PGE_2 are mediated through EP2 and EP4 receptors [5]. PGE_2 mediates bronchodilation via the EP2 receptor and most of its anti-inflammatory effects are mediated through EP2 or EP4 [6]. EP2 and EP4 receptors have also been found to be expressed in proliferative tissue in the retina [7].

Inflammatory pathways activated by PGE_2 have also been implicated in the formation of PVR membranes [8]. The EP4 receptor promotes the production of vascular endothelial growth factor (VEGF) and is thereby thought to play an important role in pathological ocular angiogenesis characteristic of neovascular retinopathies such as PDR and age-related macular degeneration [9].

PGE_2 exerts both positive and negative effects on inflammation. It thus promotes inflammation induced by leukotrienes as well as inhibits the release of inflammatory mediators by monocytes-macrophages and dendritic cells [10]. We have now examined the effects of PGE_2 on collagen gel contraction mediated by mouse RPE cells exposed to TGF-β in vitro as a model for the contraction of proliferative fibrocellular membranes in PVR and PDR.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and trypsin-EDTA were obtained from Invitrogen-Gibco (Carlsbad, CA), and 24-well culture plates and 60-mm culture dishes were from Corning-Costar (Corning, NY). Native porcine type I collagen (acid solubilized) and reconstitution buffer were obtained from Nitta Gelatin (Osaka, Japan); bovine serum albumin (BSA) was from Nacalai Tesque (Kyoto, Japan); a protease inhibitor cocktail was from Sigma-Aldrich (St. Louis, MO). PGE_2 was obtained from Biomol (Plymouth Meeting, PA), and recombinant human TGF-β2 was from R&D Systems (Minneapolis, MN). Mouse monoclonal antibodies to α-SMA were from Sigma-Aldrich, and those to Smad2, to phosphorylated Smad2, and to phosphorylated paxillin were from Cell Signaling (Beverly, MA). Mouse antibodies to paxillin was from BD Biosciences Pharmingen (San Diego, CA) and those to MMP-3 was from Assay Biotechnology (Sunnyvale, CA). Horseradish peroxidase–conjugated goat secondary antibodies and ECL Plus detection reagents were from Amersham Biosciences (Little Chalfont, UK).

Isolation and Culture of Mouse RPE Cells

Mouse RPE cells were isolated as described previously [11]. The cells were maintained under a humidified atmosphere of 5% CO_2 at 37°C in culture dishes containing DMEM supplemented with 10% fetal bovine serum.

Assay of Collagen Gel Contraction

Collagen gels were prepared as described previously [12]. In brief, 24-well culture plates were coated with 1% BSA for 1 h at 37°C. Mouse RPE cells were harvested by exposure to trypsin-EDTA, washed twice with serum-free DMEM, and resuspended in the same medium. Type I collagen (3 mg/ml), 10 × DMEM, reconstitution buffer, mouse RPE cell suspension (1.1×10^7 cells/ml in DMEM), and deionized water were mixed on ice in a volume ratio of 7:1:1:0.2:1.8 (final concentration of type I collagen, 1.9 mg/ml; final cell density, 2×10^5/ml). A portion (0.5 ml) of the resulting mixture was added to each BSA-coated well of the culture plates and allowed to solidify by incubation at 37°C under 5% CO_2 for 30 min. The collagen gels were freed from the sides of the wells with a microspatula, and serum-free DMEM (0.5 ml) with or without TGF-β2 (1 ng/ml) and PGE_2 (30 or 100 μM) was then added on top of each gel. The cells were then cultured for 24 h, after which the diameter of each gel was measured with a ruler for determination of the extent of contraction.

Immunoblot Analysis

Cells in collagen gels were lysed in a solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM NaF, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, and 1% protease inhibitor cocktail, the lysates were subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane. Nonspecific sites of the membrane were blocked, and it was then incubated consecutively with primary antibodies, horseradish peroxidase–conjugated secondary antibodies, and ECL reagents.

Gelatin Zymography

Gelatin zymography was performed as described previously [12]. In brief, culture supernatants (8 μl) from collagen gel incubations were mixed with 4 μl of nonreducing SDS sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 0.002% bromophenol blue], and 5 μl of the resulting mixture were subjected to SDS-polyacrylamide gel electrophoresis in the dark at 4°C on a 10% gel containing 0.1% gelatin. The gel was then washed with 2.5% Triton X-100 for 1 h before incubation for 18 h at 37°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, and 1% Triton X-100. The gel was finally stained with Coomassie brilliant blue.

Statistical Analysis

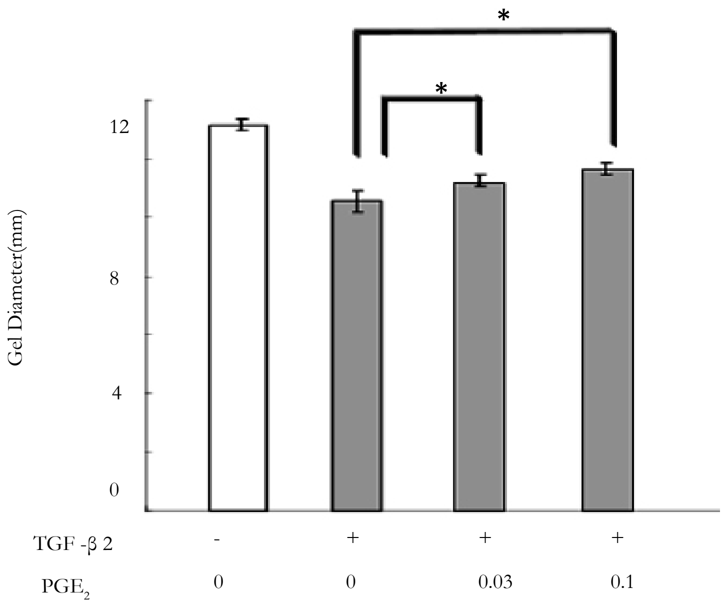
Quantitative data are presented as means ± SD and were analyzed by Dunnett's test. A *P* value of <0.05 was considered statistically significant.

Results

Effect of PGE_2 on TGF - β2 - Induced Collagen Gel Contraction Mediated by RPE Cells

We first examined the possible effect of PGE_2 on TGF-β2–induced collagen gel contraction mediated by RPE cells. The cells were thus incubated in three-dimensional collagen gels

Figure 1. Inhibition by PGE_2 of TGF-β2–induced collagen gel contraction mediated by RPE cells. Cells were incubated in three-dimensional collagen gels in the absence or presence of TGF-β2 (1 ng/ml) and the indicated concentrations of PGE_2 for 24 h, after which the extent of gel contraction was determined. Data are means ± SD of triplicates from an experiment that was repeated a total of three times with similar results. *$P < 0.05$ (Dunnett's test).



in the absence or presence of TGF-β2 and PGE_2 at various concentration for 24 h. The stimulatory effect of TGF-β2 on collagen gel contraction mediated by RPE cells was inhibited by PGE_2 in a concentration-dependent manner (Figure 1).

Effect of PGE_2 on Smad2 Phosphorylation Induced by TGF-β2 in RPE Cells

To investigate the mechanism by which PGE_2 inhibited TGF-β2–induced collagen gel contraction mediated by RPE cells, we examined the effect of this agent on the phosphorylation status of Smad2. Cells were incubated in three-dimensional collagen gels in the absence or presence of TGF-β2 and PGE_2 for 24 h, after which cell lysates were prepared and subjected to immunoblot analysis. The TGF-β2–induced phosphorylation of Smad2 was inhibited by PGE_2 (Figure 2).

Effect of PGE_2 on the Expression of α-SMA Induced by TGF-β2 in RPE Cells

We next examined the effect of PGE_2 on expression of the mesenchymal marker α-SMA induced by TGF-β2 in RPE cells. Immunoblot analysis of cells incubated in collagen gels for 24 h in the absence or presence of TGF-β2 and PGE_2 revealed that the up-regulation of α-SMA expression induced by TGF-β2 was inhibited by PGE_2 (Figure 3).

Effect of PGE_2 on Paxillin Phosphorylation Induced by TGF-β2 in RPE Cells

The effect of PGE_2 on phosphorylation of the focal adhesion protein paxillin induced by TGF-β2 was examined in RPE cells in collagen gel cultures. Incubation of the cells for 24 h with TGF-β2 increased the level of paxillin phosphorylation, and this effect was prevented by PGE_2 (Figure 4).

Effect of PGE_2 on MMP release Induced by TGF-β2 in RPE Cells

Culture supernatants obtained from RPE cells incubated in collagen gels in the absence or presence of TGF-β2 and PGE_2 for 24 h were analyzed for the release of MMPs. Gelatin zymography of the culture supernatants revealed that TGF-β2 increased the production of both MMP-2 and MMP-9 by RPE cells, and that these effects were inhibited by PGE_2 (Figure 5). Immunoblot analysis revealed that neither TGF-β2 nor PGE_2 affected the release of MMP-3 by RPE cells (Figure 5).

Discussion

We have here shown that PGE_2 inhibited the TGF-β2–induced contraction of a collagen gel mediated by mouse RPE cells. PGE_2 also inhibited the phosphorylation of Smad2 and paxillin as well as the expression of α-SMA induced by TGF-β2 in these cells. In addition, the TGF-β2–induced release of MMP-2 and MMP-9 from RPE cells was attenuated by PGE_2. These results thus suggest that PGE_2 inhibits TGF-β2–induced collagen contraction mediated by RPE cells by preventing the EMT and focal adhesion formation as well as through down-regulation of MMP expression in these cells. Moreover, these effects of PGE_2 may be mediated, at least in part, through inhibition of Smad2 signaling elicited by TGF-β2.

Proliferative tissue that forms on or under the retina during the development of PVR or PDR consists of RPE cells, fibroblasts, glial cells, and macrophages surrounded by extracellular matrix (ECM) [13]. The proliferative fibrocellular membranes formed by these cells can give rise to tractional retinal detachment. The contribution of RPE cells to these disorders is promoted by the EMT, which is characterized in part by the induction of α-SMA

Figure 2. Inhibition by PGE_2 of TGF-β2–induced Smad2 phosphorylation in RPE cells in collagen gel cultures. (a) Cells were incubated in three-dimensional collagen gels in the absence or presence of TGF-β2 (1 ng/ml) and PGE_2 (100 μM) for 24 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to total or phosphorylated (p-) forms of Smad2. Data are representative of three independent experiments. (b) Quantitative analysis of Smad2 phosphorylation. *$P < 0.05$ (Dunnett's test).

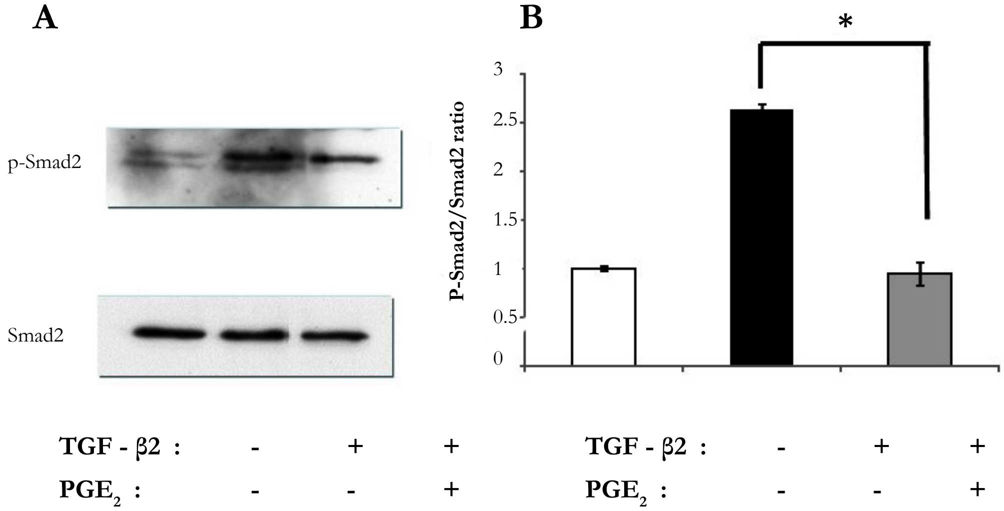


Figure 3. Inhibition by PGE_2 of TGF-β2–induced α-SMA expression in RPE cells in collagen gel cultures. (a) Cells were incubated in three-dimensional collagen gels in the absence or presence of TGF-β2 (1 ng/ml) and PGE_2 (100 μM) for 24 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to α-SMA and to β-actin (internal control). Data are representative of three independent experiments. (b) Quantitative analysis of α-SMA expression. *$P < 0.05$ (Dunnett's test).

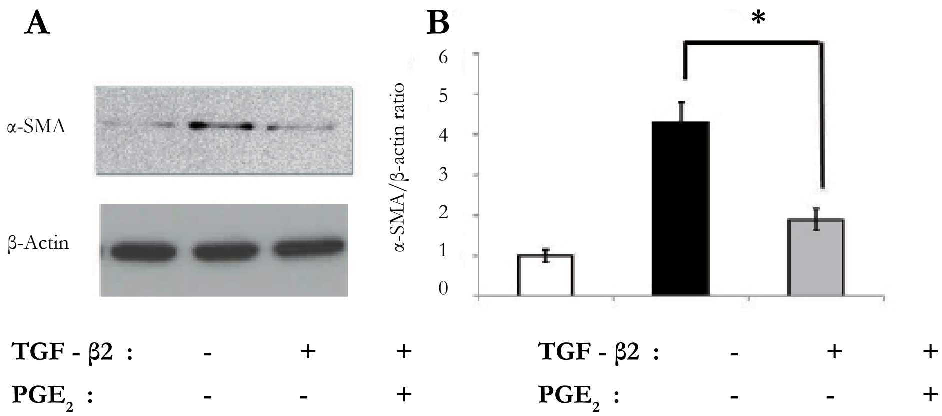


Figure 4. Inhibition by PGE_2 of TGF-β2–induced paxillin phosphorylation in RPE cells in collagen gel cultures. (a) Cells were incubated in three-dimensional collagen gels in the absence or presence of TGF-β2 (1 ng/ml) and PGE_2 (100 μM) for 24 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to total or phosphorylated (p-) forms of paxillin. Data are representative of three independent experiments. (b) Quantitative analysis of paxillin phosphorylation.*$P < 0.05$ (Dunnett's test).

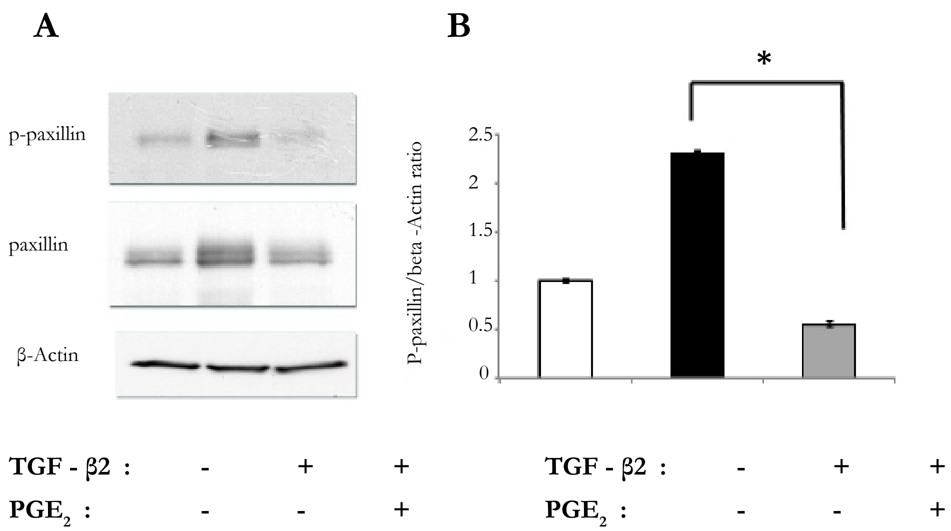
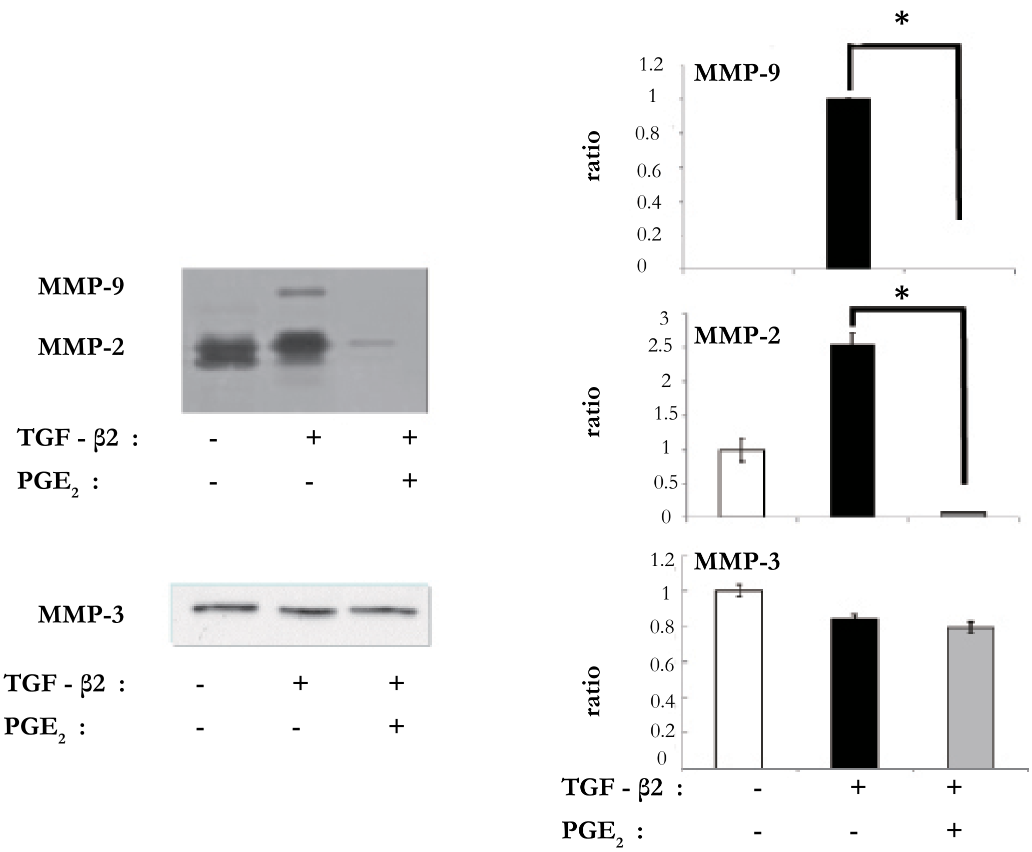


Figure 5. Inhibition by PGE_2 of TGF-β2–induced MMP release by RPE cells in collagen gel cultures. (a) Cells were incubated in three-dimensional collagen gels in the absence or presence of TGF-β2 (1 ng/ml) and PGE_2 (100 μM) for 24 h, after which culture supernatants were subjected either to gelatin zymography for detection of MMP-2 and MMP-9 or to immunoblot analysis with antibodies to MMP-3. Data are representative of three independent experiments. (b) Quantitative analysis of MMP release.*$P < 0.05$ (Dunnett's test).



and fibronectin and the acquisition of contractile properties [14]. We have now shown that PGE_2 inhibited the TGF-β2–induced expression of α-SMA in, as well as collagen gel contraction mediated by, RPE cells. Interactions with the ECM play a key role in the generation of mechanical force during cell contraction [15].

MMPs mediate remodeling of the ECM and thereby contribute to wound healing in the eye as well as to retinal disease, with MMP-2 and MMP-9 having been implicated in the pathogenesis of diabetic retinopathy [16]. MMP-2 has also been shown to play a role in the generation of mechanical stress in RPE cells [17]. We found that the release of MMP-2 and MMP-9 induced by TGF-β2 in RPE cells was inhibited by PGE_2. Moreover, we found that a MMP-specific inhibitor suppressed the TGF-β2–induced contraction of RPE cells [18]. These results thus suggest that PGE_2 attenuates induction of the EMT as well as MMP expression by TGF-β2 in RPE cells, and that it might therefore also inhibit the formation or contraction of proliferative fibrocellular membranes in PVR or PDR.

Interactions with the ECM increase tractional force in fibroblasts and epithelial cells [19]. Tyrosine phosphorylation of paxillin induced by ECM-cell interaction contributes to the formation of focal adhesions and F-actin reorganization [20]. We have previously shown that tyrosine phosphorylation of paxillin plays role in the adhesion and migration of corneal epithelial cells [21]. Endothelin-1 was also previously shown to increase the contractility of lung fibroblasts by up-regulating the expression of α-SMA, paxillin, ezrin, and moesin [22]. In addition, we previously found that the phosphorylation of paxillin is induced during collagen gel contraction mediated by cultured corneal fibroblasts [23]. Our present results show that PGE_2 inhibited paxillin phosphorylation associated with the TGF-β2–induced contraction of RPE cells, suggesting that PGE_2 modulates the activation of focal adhesions by TGF-β2 in these cells, and that it might therefore inhibit the contraction of proliferative fibrocellular membranes in individuals with PVR or PDR.

PGE_2 is a key regulator of inflammation. However, whereas PGE_2-EP4 signaling promotes immune inflammation in various diseases, PGE_2 also suppresses the production of inflammatory cytokines by dendritic cells and appears to exert anti-inflammatory effects [24]. The production of PGE_2 is up-regulated in certain ocular diseases, suggesting that this agent plays a role in these conditions [25]. We have examine that the EP4 receptor is expressed in the proliferative lesions of PVR or PDR patients (data not shown). These results suggested that this receptor might mediate the inhibitory effect of PGE_2 on TGF-β2–induced RPE cell contraction as well as a potential inhibitory effect of PGE_2 on the contraction of the proliferative fibrocellular membranes.

In summary, we have shown that PGE_2 inhibited TGF-β2–induced collagen contraction mediated by RPE cells. PGE_2 also inhibited the expression of α-SMA, the phosphorylation of paxillin, as well as the up-regulation of MMP-2 and MMP-9 in TGF-β2–stimulated RPE cells, with these effects likely resulting at least in part from attenuation of TGF-β2–induced Smad2 signaling. Our results thus suggest that PGE_2 warrants further investigation as a potential drug for the treatment of PVR, PDR, and other proliferative retinal diseases.

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