Induction by Latanoprost of Collagen Gel Contraction Mediated by Human Tenon Fibroblasts: Role of Intracellular Signaling Molecules

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PURPOSE. The outcome of glaucoma filtration surgery is affected by subconjunctival wound healing. The effects of the antiglaucoma drug latanoprost on the contractility of human Tenon fibroblasts (HTFs) cultured in a three-dimensional collagen gel were investigated.

METHODS. HTFs were cultured in a type I collagen gel with latanoprost or various inhibitors of intracellular signaling. Collagen gel contraction was evaluated by measurement of gel diameter, and collagen degradation was determined by measurement of the amount of hydroxyproline generated by acid-heat hydrolysis of culture supernatants. Phosphorylation of mitogen-activated protein kinases (MAPKs), focal adhesion kinase (FAK), and myosin light chain (MLC) was assessed by immunoblot analysis, and the formation of actin stress fibers was examined by laser confocal microscopy.

RESULTS. HTF-mediated collagen gel contraction was stimulated by latanoprost in a concentration- and time-dependent manner. Latanoprost had no effect on collagen degradation by HTFs. Latanoprost induced phosphorylation of MAPKs (ERK, p38, and JNK) and FAK, as well as the formation of stress fibers in HTFs. Furthermore, latanoprost-induced collagen gel contraction was reduced by inhibitors of ERK (PD98059 and ERK inhibitor II), p38 (SB203580), JNK (JNK inhibitor II), Rho- associated kinase (Y27632), phospholipase C (U73122), and MLC kinase (ML-7).

CONCLUSIONS. Latanoprost induced collagen gel contraction mediated by HTFs. This action of latanoprost appeared to depend on the formation of stress fibers and the activation of MAPKs, FAK, Rho-associated kinase, phospholipase C, and MLC kinase in HTFs. Latanoprost may therefore influence subconjunctival wound healing by affecting the contractility of Tenon fibroblasts. (Invest Ophthalmol Vis Sci. 2008;49:1429–1436) DOI:10.1167/iovs.07-0451

The success of filtration surgery for the treatment of glaucoma depends on the wound-healing response at the subconjunctival filtering bleb site.1 This response is mediated by a cascade of cellular and biochemical events that lead to the activation, migration, and proliferation of local fibroblasts, as well as the synthesis of new extracellular matrix (ECM) and its subsequent contraction.2 Excessive contraction of subconjunctival tissue at the wound site may result in scar formation, which in turn can lead to inadequate control of intraocular pressure and is thought to be the principal cause of surgery failure.3,4

Long-term topical antiglaucoma therapy is considered a risk factor for postoperative scarring after trabeculectomy.5 Clinical studies have thus shown that patients with a history of preoperative topical therapy have a less favorable long-term outcome of filtering surgical procedures than those without such a history.6,7 Various studies have investigated the effects of antiglaucoma drugs on Tenon fibroblasts,8,9 which play an essential role in wound healing after glaucoma filtration surgery. The antiglaucoma drug latanoprost, a prostaglandin F2α analogue, increases contraction of the bovine iris sphincter and corneal fibroblasts.10,11 Prostaglandin F2α also induces the contraction of various other cell types, including circular smooth muscle cells of the esophageal sphincter and aortic smooth muscle cells.12,13 These observations suggest that latanoprost may influence subconjunctival wound healing by affecting the contractility of Tenon fibroblasts.

Contraction of collagen gels mediated by fibroblasts has been studied as a model of cell-mediated wound contraction. Fibroblasts seeded in a gel of type I collagen adopt a morphology and arrangement similar to those apparent in vivo.14 We previously showed that latanoprost induced collagen gel contraction mediated by corneal fibroblasts.11 With the use of this model system, we have now examined the effect of latanoprost on collagen gel contraction mediated by human Tenon fibroblasts (HTFs). The effects of latanoprost on the formation of stress fibers and on the phosphorylation of the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK) as well as on that of focal adhesion kinase (FAK) and myosin light chain (MLC) in HTFs were investigated.

METHODS

Materials

Eagle’s minimum essential medium (10X), fetal bovine serum (FBS), and trypsin-EDTA were obtained from Invitrogen-Gibco (Rockville, MD), and 24-well culture plates and cell culture flasks were from Corning Corp. (Corning, NY). Native porcine type I collagen (acid solubilized) and reconstitution buffer were obtained from Nitta Gelatin (Osaka, Japan); bovine serum albumin (BSA) and methyl acetate were from Nacalai Tesque (Kyoto, Japan); protease inhibitor cocktail, collagenase, and antibodies to MLC were from Sigma-Aldrich (St. Louis, MO); and the filters (Ultrafree-MC) were from Millipore (Bedford, MA). Antibodies to ERK1 or -2 (ERK1/2), phosphorylated ERK1/2, ERK5, phospho-ERK5, p38, phospho-p38, JNK, phospho-JNK, phospho-MLC, and phospho-FAK (phospho-Tyr570/577) were obtained from Cell Sig-
Isolation and Culture of HTFs

Human subconjunctival Tenon fibroblasts were obtained with informed consent from individuals undergoing strabismus surgery. The human tissue was used in strict accordance with the tenets of the Declaration of Helsinki. The patients had no history of systemic or conjunctival diseases and did not take any topical ocular medications. The subconjunctival tissue was digested for 1 hour at 37°C with collagenase (2 mg/mL) to provide a suspension of Tenon fibroblasts. The cells from each patient were cultured separately in MEM supplemented with 10% FBS, and they were used for the present study after three to eight passages.

Assay of Collagen Gel Contraction

Collagen gels were prepared as described previously. In brief, 24-well culture plates were coated with 1% BSA for 1 hour at 37°C. HTFs were harvested by exposure to trypsin-EDTA, washed twice with serum-free MEM, and resuspended in serum-free MEM. Type I collagen (3 mg/mL; final cell density, 2 × 10^5/mL). A portion (0.5 mL) of the mixture was added to each BSA-coated well of the culture plates and allowed to solidify by incubation at 37°C under 5% CO2 for 1 hour. The collagen gels were freed from the sides of the wells with a microspatula, and serum-free MEM (0.5 mL) containing latanoprost was then added on top of each gel. For evaluation of the effects of various inhibitors on latentoprost-induced collagen gel contraction, the cells cultured in the collagen gels were incubated first for 1 hour with inhibitor and then for an additional 3 days in the same medium supplemented with latanoprost. The diameter of the gels was measured daily with a ruler, and the extent of gel contraction was calculated by subtracting the diameter at each time point from the initial diameter.

Assay of Collagenolytic Activity

Measurement of degraded collagen in culture supernatants was performed as previously described. In brief, the supernatants from collagen gel incubations were collected, and native collagen fibrils with a molecular size of >100 kDa were removed by ultrafiltration. The filtrate was then subjected to hydrolysis with 6 M HCl for 24 hours at 110°C. The amount of hydroxyproline in the hydrolysate was measured spectrophotometrically, and the extent of collagen degradation was expressed as micrograms of hydroxyproline per well.

Immunoblot Analysis

Immunoblot analysis was performed as described. HTFs were cultured in collagen gels for 24 hours in MEM supplemented with 0.5% FBS and then for an additional 24 hours in serum-free medium. They were then treated with the indicated concentrations of latanoprost for various times at 37°C, after which the gels and embedded cells were transferred to ice-cold lysis buffer (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 Na2VO4, 1% protease inhibitor cocktail). Cell lysates were centrifuged at 15,000g for 10 minutes at 4°C, and the resultant supernatants were mixed with an equal volume of 2× SDS sample buffer (250 mM Tris·HCl [pH 6.8], 2% SDS, 50% glycerol, 0.01% bromophenol blue, 10% β-mercaptoethanol) and boiled for 5 minutes. Debris was removed by centrifugation, and the samples (20 μg of protein) were then subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel. The separated proteins were transferred electrophoretically to a nitrocellulose membrane, nonspecific sites of which were blocked before incubation with antibodies to MAPKs, to FAK, to MLC, or to phosphorylated forms of these proteins (each at a 1:1000 dilution). Immune complexes were then detected with the use of secondary antibodies and enhanced chemiluminescence reagents.

Fluorescence Microscopy

A suspension of HTFs in type I collagen was prepared as described earlier, and 100-μL portions of the suspension were spread on glass-bottomed dishes that had been coated with 1% BSA. After induction of collagen gel formation, the gel was freed from the bottom of the dish and overlaid with 2 mL of MEM in the absence or presence of inhibitors. The cells were incubated for 1 hour and were then cultured for an additional 2 days in the absence or presence of latanoprost. They were then fixed for 30 minutes at room temperature with 1% paraformaldehyde in phosphate-buffered saline (PBS), washed with PBS, air-dried, and permeabilized for 30 minutes with 1% Triton X-100 in PBS. After blocking of nonspecific sites with 1% BSA in PBS, the cells were incubated first for 40 minutes with Alexa Fluor 568–conjugated phalloidin (1:200 dilution in PBS containing 1% BSA) to stain F-actin and then for 10 minutes with TOTO-3 iodide (1:2000 dilution in PBS containing 1% BSA of a 1 mM solution in dimethyl sulfoxide) to stain nuclei. The cells were examined with a laser-scanning confocal microscope (Axiovert200M; Carl Zeiss Meditec, Tokyo, Japan).

Statistical Analysis

Data are presented as the mean ± SD. All experiments were performed in triplicate and repeated at least three times. Statistical analysis was performed with the Dunnett multiple comparison test, the Tukey-Kramer test, or Student’s unpaired t test. P < 0.05 was considered statistically significant.
Effects of Latanoprost on MAPK Phosphorylation in HTFs in Collagen Gel Cultures

To investigate the possible role of MAPKs in latanoprost-induced collagen gel contraction mediated by HTFs, we first examined the effects of this drug on the activation status of ERK1/2, ERK5, p38, and JNK. Immunoblot analysis revealed that the abundance of ERK1/2, ERK5, p38, and JNK was not affected by latanoprost, whereas this drug (10 µM) induced the phosphorylation (activation) of these MAPKs in a time-dependent manner (Fig. 2). The effect of latanoprost on the phosphorylation of each MAPK was at its maximum at ~5 minutes after the onset of stimulation and persisted for up to 2 hours. The activation of ERK1/2, p38, and JNK induced by latanoprost was also concentration dependent, with the maximum increases in the phosphorylation of these MAPKs being apparent at latanoprost concentrations of 10, 100, and 100 µM, respectively (Fig. 3). The latanoprost carrier methyl acetate had no effect on the phosphorylation of these MAPKs in HTFs (data not shown).

Effects of MAPK Inhibitors on Latanoprost-Induced Collagen Gel Contraction Mediated by HTFs

We next examined whether MAPK inhibitors affected latanoprost-induced collagen gel contraction mediated by HTFs. Cells were incubated with 10 or 30 µM PD98059 (ERK inhibitor), 10 µM SB203580 (p38 inhibitor), 5 µM JNK inhibitor II, or 10 µM ERK inhibitor II for 1 hour before exposure to 10 µM latanoprost for 3 days. The stimulatory effect of latanoprost on collagen gel contraction mediated by HTFs was inhibited by 27%, 46%, 81%, or 47% in the presence of PD98059 (30 µM), SB203580, JNK inhibitor II, or ERK inhibitor II, respectively (Fig. 4). These MAPK inhibitors had no effect on HTF-mediated collagen gel contraction in the absence of latanoprost.

Effect of a Rho-Associated Kinase Inhibitor on Latanoprost-Induced Collagen Gel Contraction Mediated by HTFs

To investigate whether Rho-associated kinase might contribute to latanoprost-induced collagen gel contraction mediated by HTFs, we incubated cells with the Rho-associated kinase inhibitor Y27632 (5 µM) for 1 hour before treating them with 10 µM latanoprost for 3 days. The stimulatory effect of latanoprost on collagen gel contraction mediated by HTFs was inhibited by 27%, 46%, 81%, or 47% in the presence of PD98059 (30 µM), SB203580, JNK inhibitor II, or ERK inhibitor II, respectively (Fig. 4). These MAPK inhibitors had no effect on HTF-mediated collagen gel contraction in the absence of latanoprost.

Effects of MAPK or Rho-Associated Kinase Inhibitors on Latanoprost-Induced Stress Fiber Formation in HTFs Cultured in Collagen Gels

Given that the cytoskeleton plays an important role in cell motility, we investigated the possible effect of latanoprost on stress fiber formation in HTFs cultured in collagen gels in the absence or presence of various inhibitors. HTFs cultured in three-dimensional collagen gels for 2 days without latanoprost exhibited a dendritic morphology, with no prominent stress fibers (Fig. 5A). The presence of 10 µM latanoprost induced cell spreading, and the formation of pronounced stress fibers (Fig. 5B). This stimulatory effect of latanoprost on stress fiber formation was inhibited by PD98059 (Fig. 5C), SB203580 (Fig.
5D), JNK inhibitor II (Fig. 5E), and Y27632 (data not shown). These inhibitors had no effect on stress fiber formation in HTFs cultured without latanoprost (data not shown).

Effect of Latanoprost on FAK Phosphorylation in HTFs in Collagen Gel Cultures

To investigate the possible role of FAK in latanoprost-induced collagen gel contraction mediated by HTFs, we examined the effect of this drug on the phosphorylation status of FAK. Immunoblot analysis revealed that latanoprost (10 μM) induced a time-dependent increase in the phosphorylation of FAK, which was first apparent at 2.5 minutes, was at a maximum at 2 hours, and persisted for 48 hours after the onset of stimulation, whereas the abundance of FAK was not affected by latanoprost (Fig. 6).

Effect of PLC Inhibition on Latanoprost-Induced Collagen Gel Contraction Mediated by HTFs

We then assessed the possible role of phospholipase C (PLC) in latanoprost-induced collagen gel contraction mediated by HTFs. We incubated the cells with the PLC inhibitor U73122 (5 μM) for 1 hour before treatment with 10 μM latanoprost for 3 days. The stimulatory effect of latanoprost on collagen gel contraction mediated by HTFs was completely inhibited in the presence of U73122 (Fig. 7). Gel contraction in the absence of latanoprost was also inhibited by U73122.

Role of MLC Phosphorylation in Latanoprost-Induced Collagen Gel Contraction

We finally examined the possible role of MLC phosphorylation in latanoprost-induced collagen gel contraction mediated by
HTFs. Immunoblot analysis revealed that culture of the cells for up to 3 days in the presence of latanoprost (10 μM) had no effect on the phosphorylation of MLC (Fig. 8A). In contrast, incubation of the cells with the MLC kinase inhibitor ML-7 (10 μM) for 1 hour before culture for 3 days with or without 10 μM latanoprost resulted in complete inhibition of HTF-mediated collagen gel contraction regardless of the absence or presence of latanoprost (Fig. 8B).

**DISCUSSION**

We have shown that latanoprost induced the contraction of HTFs embedded in a type I collagen gel without affecting collagen degradation by these cells. Latanoprost also induced the formation of stress fibers and the phosphorylation of MAPKs and FAK in HTFs cultured in collagen gels. Furthermore, latanoprost-induced collagen gel contraction mediated by HTFs was inhibited by inhibitors of MAPKs, Rho-associated kinase, PLC, or MLC kinase.

Latanoprost, a prostaglandin F$_{2\alpha}$ derivative, binds with high affinity to the prostaglandin F$_{2\alpha}$ receptor, which has been shown to be abundant in the conjunctiva by immunohistochemical analysis. The MAPK cascade is a pivotal intracellular signaling module and is activated by the prostaglandin F$_{2\alpha}$ receptor. Latanoprost promotes the survival of neuroglia in the retina by interacting with the prostaglandin F$_{2\alpha}$ receptor and activating the ERK1/2 signaling pathway. Prostaglandin F$_{2\alpha}$ activates both ERK1/2 and p38 signaling pathways in smooth muscle cells of the iris sphincter. In the present study, latanoprost induced activation of the MAPKs ERK1/2, p38, and JNK in HTFs in a time- and concentration-dependent manner. Consistent with these findings, latanoprost-induced

**FIGURE 5.** Latanoprost-induced stress fiber formation in HTFs and the effects of MAPK inhibitors. Cells embedded in collagen gels were incubated first for 1 hour in the absence (A, B) or presence of 40 μM PD98059 (C), 20 μM SB203580 (D), or 5 μM JNK inhibitor II (E) and then for 2 days in the additional absence (A) or presence (B-E) of 10 μM latanoprost. They were then fixed, permeabilized, and stained with Alexa Fluor 568–labeled phalloidin (for F-actin, red) and TOTO-3 iodide (for nuclei, blue). Data are representative of results of three independent experiments. Scale bar, 50 μm.

**FIGURE 6.** Effect of latanoprost on FAK phosphorylation in HTFs cultured in collagen gels. Cells embedded in collagen gels were cultured for 24 hours in MEM supplemented with 0.5% FBS and then for an additional 24 hours in serum-free medium. They were then incubated with 10 μM latanoprost in serum-free medium for the indicated times, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to FAK or to phosphorylated (p-) FAK. Data are representative of three independent experiments.
collagen gel contraction mediated by HTFs was markedly suppressed by the MAPK inhibitors PD98059 (a specific inhibitor of ERK1/2 activation), SB203580 (a selective p38 MAPK inhibitor), or JNK inhibitor II, confirming that MAPKs play an important role in the latanoprost-induced increase in HTF contractility. Given that high concentrations of PD98059 may inhibit additional members of the MAPK family such as ERK5,22 we also tested the effect of ERK inhibitor II, a more specific inhibitor of ERK1/2 activation, to be certain of the specificity of the observed effect of PD98059. ERK inhibitor II also inhibited latanoprost-induced collagen gel contraction mediated by HTFs. However, our observation that latanoprost also induced phosphorylation of ERK5 in HTFs cultured in collagen gels suggests that activation of both ERK1/2 and ERK5 may contribute to latanoprost-induced HTF contraction.

The contraction of fibroblasts is mediated by a pronounced intracellular cytoskeletal system and is thought to be responsible for wound closure during wound healing. The formation of actin stress fibers contributes to collagen gel contraction mediated by fibroblasts.16–22 Prostaglandin F2α induces both the formation of actin stress fibers and changes in morphology in 293-EBNA cells.24 We have now shown that latanoprost induced the formation of stress fibers in HTFs in a manner sensitive to MAPK inhibitors, suggesting that this effect contributes to the latanoprost-induced increase in HTF contractility. The polymerization and reorganization of actin microfilaments and associated actin stress fiber formation require the small GTPase Rho,53 which has also been shown to regulate the contractility of HTFs.20 In the present study, an inhibitor of Rho-associated kinase inhibited latanoprost-induced HTF contraction as well as stress fiber formation in these cells, suggesting that the latanoprost-induced increase in HTF contractility may also be mediated by activation of Rho-associated kinase. The prostaglandin F2α receptor activates a Rho signaling pathway that leads to phosphorylation of FAK,24 a protein tyrosine kinase that plays an important role in the assembly of focal adhesions and cell contractility.27 We have now shown that latanoprost induced activation of FAK in HTFs, suggesting that this effect may contribute to the latanoprost-induced increase in HTF contractility. Rho-associated kinase also activates PLC-dependent signaling pathways via the heterotrimeric GTP-binding protein Gq, resulting in an increase in intracellular Ca2+ concentration and the activation of protein kinase C.28 In the present study, U73122 (a specific inhibitor of PLC activation) blocked latanoprost-induced collagen gel contraction mediated by HTFs, implicating PLC signaling in this action of latanoprost.

The time scale of MAPK activation induced by latanoprost (minutes to hours) was smaller than that of HTF-mediated collagen gel contraction induced by this drug (days). However, given that MAPK inhibitors inhibited latanoprost-induced gel contraction, it is possible that an initial activation of MAPKs is important for triggering other intracellular signaling events that underlie long-term HTF contraction.

An increase in the intracellular Ca2+ concentration and the subsequent phosphorylation of MLC by Ca2+-calmodulin-dependent MLC kinase play an important role in regulation of actomyosin contractility,31,32 as does modulation of the Ca2+ sensitivity of the contractile apparatus.31,32 Both MLC phosphorylation-dependent and independent Ca2+ sensitization have been described. Prostaglandin F2α, but not latanoprost, was found to increase the Ca2+ sensitivity of the pig iris sphincter muscle in an MLC phosphorylation-dependent manner.33,34 The lack of apparent effect of latanoprost on MLC phosphorylation in HTFs in the present study suggests that MLC phosphorylation-independent Ca2+ sensitization may underlie the latanoprost-induced contraction of these cells. However, we found that ML-7 (a selective inhibitor of MLC kinase) prevented HTF-mediated collagen gel contraction regardless of the absence or presence of latanoprost, suggesting that MLC phosphorylation is necessary for both basal and stimulated HTF contraction. The reason for this apparent discrepancy in our results remains to be determined.

Latanoprost may limit net ECM production by Tenon fibroblasts through stimulation of the release of matrix metalloproteinases (MMPs) from these cells.35 Topical application of prostaglandin F2α reduces the amounts of collagen types I, III, and IV in the monkey uveoscleral outflow pathway.35 Specific prostaglandins have also been shown to increase the release of MMPs from ciliary smooth muscle cells.36 Cell migration is facilitated by MMP-mediated degradation of components of the surrounding ECM. Inhibition of MMPs has been shown to limit subconjunctival scarring after experimental glaucoma filtration surgery in the rabbit by reducing the number of migrating fibroblasts.1 In our experimental system, however, latanoprost had no effect on collagen degradation mediated by HTFs.

Trabeculectomy is the most frequently performed surgical procedure for the treatment of glaucoma.39 However, the contraction of Tenon fibroblasts during subconjunctival wound healing results in scar formation at the filtering bleb site,2–40 which is thought to be the main cause of the failure of glaucoma filtration surgery.2 Long-term topical antiglaucoma therapy has been implicated as a risk factor in postoperative scarring after trabeculectomy.3 Latanoprost is currently prescribed as a first-line drug for glaucoma therapy, and trabeculectomy in eyes treated after surgery with latanoprost has been found to result in a smaller decrease in intraocular pressure...
than in eyes naive to such treatment. In the present study, latanoprost stimulated collagen gel contraction mediated by HTFs, suggesting that the application of latanoprost eyedrops may affect subconjunctival wound healing by increasing the contractility of Tenon fibroblasts. The results obtained with our in vitro model cannot be fully extrapolated to in vivo conditions, however, given that HTFs in our experiments were exposed continuously for >24 hours to medium containing a fixed concentration of latanoprost. In contrast, eyedrops are rapidly diluted after their administration. However, long-term application of antiglaucoma eyedrops is necessary to maintain intraocular pressure within the normal range in some patients. The effect of latanoprost eyedrops on the contractility of Tenon fibroblasts and the consequent potential impact on subconjunctival wound healing after filtration surgery should thus be borne in mind when this drug is administered for prolonged periods.

In summary, our results suggest that latanoprost promotes the contraction of Tenon fibroblasts in a manner dependent on the formation of stress fibers and the activation of MAPKs, FAK, Rho-associated kinase, PLC, and MLCK. Latanoprost may therefore contribute to scar formation during subconjunctival wound healing after filtration surgery by affecting the contractility of Tenon fibroblasts. Further characterization of the effects of latanoprost or other antiglaucoma drugs on Tenon fibroblasts in vitro and in vivo as well as on the outcome of glaucoma filtration surgery is warranted.

References


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