Effects of Prostaglandin Analogues on Human Ciliary Muscle and Trabecular Meshwork Cells

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Purpose. To determine the effects of prostaglandin $F_{2\alpha}$ analogues on gene expression of human ciliary muscle (HCM) and trabecular meshwork (HTM) cells.

Methods. Cultures of HCM and HTM cells were established from five different donors treated for 9 days with 10 μg/mL of either latanoprost (free acid) or prostaglandin $F_{2\alpha}$ ethanolamide and compared with control cells. The mRNA from the cells of the five individual donors was pooled and analyzed by using gene microarrays. Gene expression changes were confirmed by either real-time PCR or relative quantitative PCR.

Results. Approximately 12 genes showed a twofold or greater change in expression under experimental conditions. Four of these may alter outflow. Aquaporin-1 and versican were downregulated in the HCM, whereas *IGF1* and fibroleukin were upregulated in HTM. Expression levels of *TNFSF10* and promelanosome-concentrating hormone also increased in the treated HTM cells. The mRNA levels for the prostaglandin FP receptor were downregulated in the ciliary muscle cells. Optineurin and αB-crystallin levels remained unchanged, but myocilin in the HTM cells was decreased in some samples.

Conclusions. Both analogues changed gene expression similarly in either HCM or HTM cells, but the changes appeared to be cell specific, perhaps indicating that other transcription factors are influential. Outflow of aqueous humor may be increased by the prostaglandin analogues by alterations in the extracellular matrix. Other changes may influence cellular metabolism, such as the increases in *IGF1*, tumor necrosis factor superfamily-10 and promelanosome-concentrating hormone. (*Invest Ophthalmol Vis Sci.* 2003;44:1945–1952) DOI: 10.1167/iovs.02-0920

Recently developed prostaglandin analogues appear to be efficacious and are well-tolerated agents for the reduction of intraocular pressure (IOP) in patients with primary open-angle glaucoma (POAG) and ocular hypertension.[1] Latanoprost (free acid) is the active form of a prostaglandin $F_{2\alpha}$ analogue. This drug decreases IOP by increasing aqueous outflow through the uveoscleral outflow system.[2,3] Compounds related to prostaglandins called prostamides are also used to reduce IOP.[4] These drugs, which have structures similar to those of prostaglandins, are thought to enhance outflow through both the uveoscleral outflow pathway and the traditional outflow system, but the actual mechanisms of their action are still unknown. Trabecular meshwork and ciliary muscle are two major tissues of the outflow systems, but little is known about the biological changes in these two tissues during long-term use of prostaglandin analogues.

This study was undertaken to investigate the changes in mRNA expression in the human trabecular meshwork (HTM) and ciliary muscle (HCM) cells when treated with latanoprost and prostaglandin $F_{2\alpha}$ ethanolamide (prostamide) for 9 days. We tried to determine what longer-term changes might be happening in these two cell types, as opposed to the more acute changes that could occur when the compounds are initially added to the cells. The dosage chosen (10 μg/mL) was selected because this concentration was the one that showed effects on some melanoma cells in a previous report investigating the effects of latanoprost on tyrosinase activity and mitotic index,[5] although this is a larger dose than used therapeutically. With this study, we wanted to determine any differences in the two types of compounds and to discover genes that might explain the actions of prostaglandin on outflow.

Materials and Methods

Cells Culture and Drug Treatment

Five pairs of normal human eyes from donors (ages: 30, 66, 73, 76, and 86 years) with no history of eye diseases were obtained from the National Disease Research Interchange (Philadelphia, PA) around 30 to 36 hours after death. Samples of HTM and HCM were dissected as previously described.[6–8] Briefly, the globes were placed in phosphate-buffered saline (PBS) at room temperature for 15 minutes on arrival and were cut into two parts through the ora serrata with a sterile blade. The lens and iris were gently removed from the anterior segment. The HTM and HCM were carefully isolated from the surrounding tissues. The tissue was cut into small sections, and the cells were cultured at 37°C in a 10% CO_2 atmosphere in Dulbecco's modified Eagle's medium with 20% fetal bovine serum, 2 mM L-glutamine, and 0.25 μg/mL gentamicin (GibcoBRL Life Technologies, Gaithersburg, MD). After 2 weeks, nonadherent cells were removed and trypsinized with 0.25% trypsin and 1 mM EDTA for 10 minutes. The cells were centrifuged and replated into Petri dishes. At the next change of medium, all debris in the culture medium was discarded. Ten human primary cell cultures (five HTM and five HCM) from the five different eyes were used in this experiment. Three flasks of confluent cells from each eye, at fewer than five passages, were treated with vehicle, 0.1% ethanol (control), 10 μg/mL latanoprost, or 10 μg/mL prostaglandin $F_{2\alpha}$ ethanolamide (prostamide) every other day, or four times in a 9-day period. A small number of HCM cells were also plated on microscope slides for immunofluorescent studies.

RNA Isolation and Purification

Total RNA was isolated from the cells for each of the experimental conditions with extraction reagent (TRIzol; GibcoBRL Life Technologies). The media of the culture cells was completely removed and the cells were immediately rinsed with PBS followed by cell lysis after adding the appropriate amount of reagent (1 mL/10 cm^2 surface area) directly to the culture flask. The cells and cell lysates were scraped from the bottom of the culture flask with a cell scraper. RNA was

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TABLE 1. Human Specific Primer Sequences and Sizes of Real-Time RT-PCR Product

Gene		5′–3′ Sequence	Amplicon Size (bp)	Expect Value
Myocilin	F	5′-cgactaaggcaagaaaatgagaatc-3′	76	e-144
	R	5′-cccttctcagccttgctacct-3′		
Optineurin	F	5′-gagaaggctctggcttccaa-3′	86	5e-70
	R	5′-gtcatggtttccaggtcctctt-3′		
αB-crystallin	F	5′-tggaccaaggaaacaggtctct-3′	74	7e-91
	R	5′-cggtgacagcaggcttctct-3′		

F, forward; R, reverse.

prepared according to the protocol from the manufacturer. The RNA pellets were washed with 75% ethanol, centrifuged, and dried. The residual DNA was removed by treatment with DNase I. Pellets were resuspended in 30 μL of diethyl pyrocarbonate (DEPC)–treated water, followed by the addition of 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 20 U RNase-free DNase I (Roche Molecular Biochemicals, Indianapolis, IN), and 20 U RNasin (Promega Corp., Madison, WI) in a total volume of 60 μL. Samples were incubated at 37°C for 25 minutes. Then the RNA was cleaned with a kit (RNeasy Mini Kit; Qiagen, Valencia, CA) according to the protocol of the manufacturer. RNA concentration and purity were determined by measuring optical density at 260 and 280 nm with a spectrophotometer. RNA integrity was monitored by 2% agarose gel (E-Gel; Invitrogen, Carlsbad, CA) electrophoresis with the 28S and 18S RNA bands, stained with ethidium bromide, visualized by exposure to UV light.

Gene Microarray Analysis

The same amount of total RNA from each of the five eyes (2 μg) was taken and pooled to generate six samples (HTM-control, HTM-latanoprost, HTM-prostamide, HCM-control, HCM-latanoprost, HCM-prostamide). The pooled samples were precipitated and quantified again for cDNA synthesis. Doubled-stranded cDNA was synthesized from 10 μg purified total RNA (Superscript Choice system; Gibco BRL Life Technologies) and a T7-(dT)24 primer (Genset, La Jolla, CA). After double-stranded cDNA was purified by phenol-chloroform extraction, in vitro transcription reactions were performed with an RNA transcript labeling kit (Bioassay High Yield RNA Transcript Labeling Kit; Enzo Diagnostics, Farmingdale, NY) according to the manufacturer's protocol. Biotin-labeled cRNA was purified (Qiagen) and quantified before being fragmented to 35- to 200-base fragments. Duplicate chips for each of the six samples were formed starting with the same total RNA. Twelve gene microarrays (HG-U95Av2 Genechips; Affymetrix, Santa Clara, CA) were used. Washing, staining, and scanning were performed by using the instrument system accompanying the microarrays (Genechip Instrument System; Affymetrix), as recommended in the manufacturer's technical manual. The arrays were scanned and data were analyzed using the software provided (Microarray Suite Software; Affymetrix). Both algorithms from the software versions (Empiric, ver. 4, and Statistical ver. 5; Affymetrix) were used for the analyses.

The absolute analysis results of each chip were scaled to the same target intensity and could then be directly compared with one another. The absolute analysis calculates a variety of metrics by using the probe array's hybridization intensities measured by the scanner. Some are used for background and noise calculations. The comparison analysis performs additional calculations on data from two separate probe array experiments to compare gene expression levels between two samples. The comparison analysis begins with the absolute analysis of one probe array experiment as the source of baseline data and a second probe array of the experimental condition as the source of data to be compared with the baseline. Because both experimental and control results were run twice, four comparisons for each experimental condition were determined (two control duplicates compared separately with two experimental duplicates). Those genes that had increased or decreased expression greater than twofold in all four comparisons were considered for additional verification.

Real-Time PCR

cDNA was generated from the total RNA samples identical with the ones used for the chip analysis by use of a transcription kit (*Taq*man Reverse Transcription Reagents; Applied Biosystems, Foster City, CA). Primers were designed with the accompanying software (Primer Express Software, ver. 2.0; Applied Biosystems) and their concentrations optimized according to the manufacturer's protocol (SYBR Green PCR Master Mix and RT-PCR; Applied Biosystems). The purpose of optimizing primer concentrations is to determine the minimum primer concentrations that produce the lowest threshold cycle (C_T) and maximum change in normalized reporter signal (ΔRn), while minimizing nonspecific amplification. The forward and reverse primers for the human myocilin, optineurin, and αB-crystallin are found in Table 1. Real-time PCR was performed using green nucleic acid dye (SYBR Green Dye; Applied Biosystems). Direct detection of the PCR product was monitored by measuring the increase in the fluorescence generated by the binding of the green dye to double-stranded DNA (Prism 7900HT; Applied Biosystems). All PCR reactions were performed in triplicate. Relative quantitation of gene expression was determined by the standard curve method (ABI PRISM 7700 Sequence Detection System; Applied Biosystems). For comparison of transcript levels between samples, standard curves were prepared for both the target gene and the endogenous reference (18S ribosomal RNA). For each experimental sample, the amount of target and endogenous reference was determined from the appropriate standard curves. The target amount was then divided by the endogenous reference amount to obtain a normalized target value. Each of the experimental normalized sample values was divided by the normalized control sample value to generate the relative expression levels. Because of the small size of the real-time amplicons, sequencing of the products was difficult. One additional primer was made for each gene to generate DNA by RT-PCR that could be sequenced with the sequencing kit (BigDye DNA Sequencing kit; Applied Biosystems). The primers selected were myocilin reverse primer 5′-TCCACACACCATACTTGCC-3′; αB-crystallin forward primer 5′-GTTGGGAGATGTGATTGAGG-3′; and optineurin reverse primer 5′-AAACCCTGACCCCAAGTGATCC-3′. The sequences obtained from the DNA sequencer (ABI Prism 310; Applied Biosystems) were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). The BLAST expect values are reported when the expect value is the statistical probability that the match is by chance alone.

Relative Quantitative Reverse Transcription–Polymerase Chain Reaction

Relative quantitative reverse transcription–polymerase chain reaction (RQ-PCR) analyses were performed using a two-step system. The initial RNA pooled samples were identical with those used for the arrays. The reverse transcription reaction was performed with a kit (RetroScript; Ambion, Austin, TX), according to the manufacturer's protocol. For the PCR reaction, primers were designed to cross at least one intron–exon boundary of the genes of interest and the products generated were checked by using the DNA sequencing kit (BigDye DNA Sequencing; Applied Biosystems) to confirm their identity. The reaction product

TABLE 2. Human Specific Primer Sequences and Sizes of RT-PCR Products

Gene	5′–3′ Sequence	Product Size (bp)	Expect Value
Aquaporin 1	F 5′-tattgactacactggctgtggg-3′ R 5′-ccaggatgaagtcgtagatgag-3′	146	2e-49
Fibroleukin	F 5′-atgggagcaccaacttcac-3′ R 5′-acaaggcatatagttcgacac-3′	171	2e-39
Optineurin	F 5′-aaacagaaggaagaacgccag-3′ R 5′-tcagacacgatggcccaacag-3′	257	9e-90
Promelanosome-concentrating factor	F 5′-tggtctgccactcaatctg-3′ R 5′-gctgagttttcttcatccc-3′	135	9e-26
Prostaglandin receptor	F 5′-aacctgccagacggaaaac-3′ R 5′-cagaaacgatgccttggac-3′	142	2e-24
Somatimedin C	F 5′-atcagcagtcttccaaccc-3′ R 5′-gcttgttgaaataaaagcccc-3′	217	6e-81
TNFSF10 (TRAIL)	F 5′-gcagctcacataactgggac-3′ R 5′-gctcaggaatgaatgcccac-3′	129	3e-31
Versican	F 5′-ccaccactgtttcttcatttttc-3′ R 5′-aaaagccccttatttctgcc-3′	237	2e-87

F, forward; R, reverse.

was sequenced with a commercial system (ABI Prism 310; Applied Biosystems). The sequences obtained were analyzed with the BLAST program. The BLAST expect values are reported in Table 2. RQ-PCR was performed by using a kit (QuantumRNA 18S Internal Standards; Ambion), according to the supplied protocol. The number of PCR cycles, the linear range, and the competimer ratio for the 18S rRNA were established for each gene. Products were separated on 2% agarose gel (E-Gel; Invitrogen), the ethidium-bromide–stained DNA was visualized with exposure to UV light and analyzed by image-analysis software (ImageQuant; Molecular Dynamics, Amersham Biosciences, Piscataway, NJ). The ratio between the signal for the amplicon being investigated and the signal for the 18S rRNA sample was calculated. Five different reactions were performed for each sample, and the ratios for the five reactions were averaged for comparison.

Immunofluorescence

All five cultures of HCM cells were checked for the presence of both smooth muscle actin and desmin. The cells were grown on microscope slides, fixed with acetone-methanol 1:1 for 30 minutes at −20°C. Fixative was removed, and the cells were rinsed in PBS two times. After removal of the PBS, 200 μL of 10% horse serum (in PBS) was added to block the cells for 30 minutes. Primary antibody anti-α-SMA (Sigma, St. Louis, MO) with a dilution of 1:50 or anti-desmin (Oncogene, Uniondale, NY; or Chemicon, Temecula, CA) with a dilution of 1:20 was applied to the cells and incubated at room temperature for 1 hour after removal of the serum. α-SMA and desmin were visualized with fluorescein or Texas red anti-mouse IgG (H+L) secondary antibody (Vector Laboratories, Inc., Burlingame, CA), followed by rinsing six times in a 30-minute period. The stained cells were viewed under a fluorescence microscope.

RESULTS

Both the HTM cells and HCM cells grew well in tissue culture, although there were some differences in the times to yield confluent cultures in the flasks. The HCM cells from all five donors were positive for the presence of smooth muscle actin and desmin by immunofluorescence. These two proteins were chosen because they have been used to test whether a culture contains actual ciliary muscle cells.[9] The results obtained with the HCM cells from a 66-year-old donor are shown in Figure 1.

Similar results were obtained with the other HCM cells. Treatment with either latanoprost or prostamide induced no obvious morphologic changes between control cells and treated cells, as observed with the phase-contrast microscope (Fig. 2).

Analyses of the comparisons using both algorithms (Empiric and Statistical software; Affymetrix) yielded similar results, with the Empiric algorithm showing the expression of slightly more genes to be changed twofold than the newer algorithm, but the differences between the results of the two algorithms were minor. Only approximately 10 to 15 genes of the approximate 12,000 genes on the array had expression changes exceeding our stringent threshold of a twofold change, using an iterative comparison analysis strategy. Confirmation of gene expression changes was achieved with either real-time PCR or RQ-PCR. All results were verified by using the original RNA that was used for the array.

Three genes that have been reported to be associated with glaucoma or to have expression changed in glaucomatous tissue, were checked by real-time PCR. The mRNA of one of these genes, myocilin, increased twofold on the arrays for HCM cells treated with prostamide. The standard curves from the real-time PCR obtained for the myocilin and 18S rRNA stan-

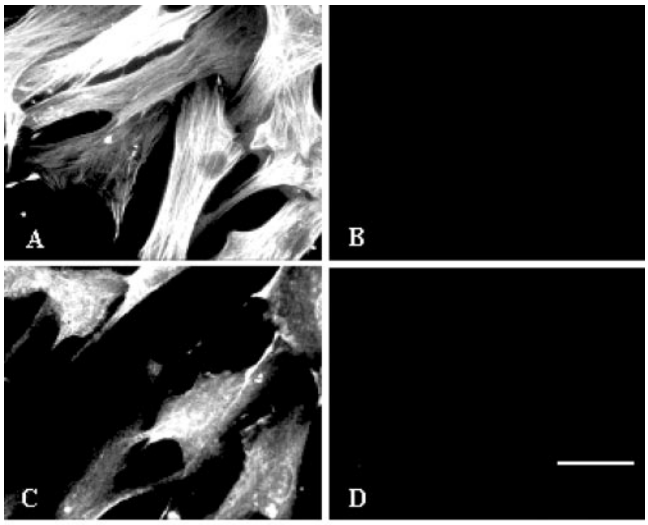

FIGURE 1. Immunofluorescence of HCM cells. HCM cells labeled with antibody to smooth muscle actin (**A**) and the negative control (**B**). HCM cells labeled with antibody to desmin (**C**) and the negative control (**D**). All images are at the same magnification. Bar: 50 μm.

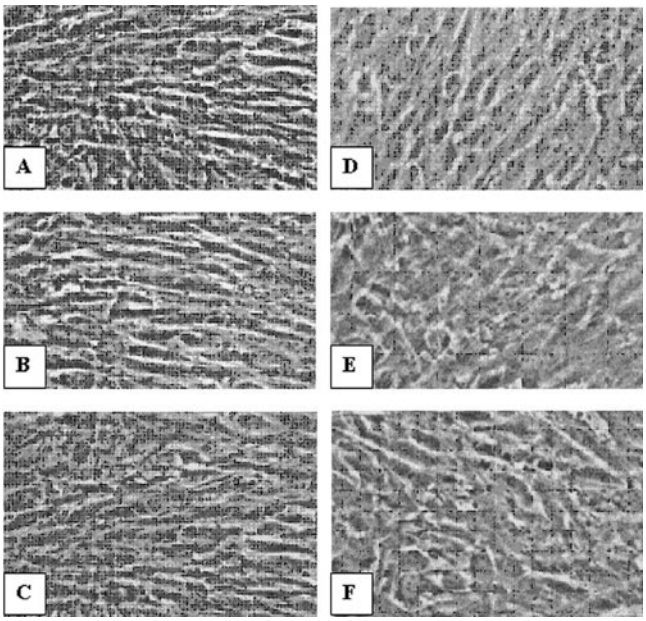

FIGURE 2. HTM and HCM showed no obvious morphologic changes between control cells and treated cells, as observed with the phase-contrast microscope. HTM control cells (**A**), HTM cells with latanoprost treatment (**B**), HTM cells with prostamide treatment (**C**), HCM control cells (**D**), HCM cells with latanoprost treatment (**E**), and HCM cells with prostamide treatment (**F**). Magnification, ×200.

dards are shown (Fig. 3) with the equation shown for each. Real-time PCR indicated that there was little change of myocilin in the HCM but that there was a decrease in the HTM pooled sample. The graphs (Fig. 4) show the results of the real-time PCR of the myocilin on HTM cells for the mRNA pooled mixture from the cells, used with the microarrays, as well as the individual mRNA samples themselves. Analysis of each of the five individual mRNA samples showed that only three of the samples had decreases, with the other two showing slight increases. This type of variation was also reported using glucocorticoid-treated HTM cells from different donors.[10]

A second gene that has been found to be associated with POAG is optineurin.[11] Oligonucleotides of this gene are present on the array. Analysis of the microarrays indicated no change in the mRNA of this gene with either prostaglandin analogue treatment. Both real-time PCR and RQ-PCR also showed no significant differences between the control and experimental samples from the pooled mRNA. Real-time PCR of the individual mRNA samples also showed no differences (data not shown).

A third protein reported to be increased with glaucoma is αB-crystallin. The expression of this gene has been shown to increase with heat shock, TGFβ treatment, oxidative stress, and glucocorticoid treatment in HTM cells.[12] Although the gene was not printed on the array, real-time PCR indicated no significant difference in control and experimental samples in either the HTM or the HCM cells using either the pooled mRNA or individual mRNA samples similar to the results from optineurin (data not shown).

A typical RQ-PCR gel used for quantitation by image-analysis software (ImageQuant; Molecular Dynamics, Amersham Biosciences) is shown for the gene fibroleukin (Fig. 5). The mRNA for this gene increased with both latanoprost (sample marked L) and prostamide (P), compared with the RQ-PCR of the control culture (C). The top stained band is the 18S rRNA at 315 bp and can be compared with the lower fibroleukin band of 171 bp. The five individual products of each treatment condition were similar to each other. After analysis by the software, the normalized magnitudes of change (± SD) were control 1.00 ± 0.21, latanoprost 2.57 ± 0.33, and prostamide 6.11 ± 0.93. These data corresponded with the increases recorded by the array of 7.3 for latanoprost and 15.0 for prostamide.

To get an indication of the consistency of the RQ-PCR method used, the change in expression of the prostaglandin FP receptor was chosen. This gene is not on the array. By RQ-PCR, the expression of the prostaglandin receptor in the HCM cells decreased approximately fourfold with addition of either latanoprost or prostamide, compared with the control cells. To check the day-to-day variability of the RQ-PCR, the assays, each consisting of five reactions, was run on three different days. The bands in the agarose gels were quantitated and the magnitudes of change of the prostaglandin receptor in the HCM were compared. With both the latanoprost- and the prostamide-treated cells, the change varied from approximately −3- to −5.5-fold (Fig. 6A). For the samples marked run 2, samples from the 30- and 73-year-old donors were assayed at the same

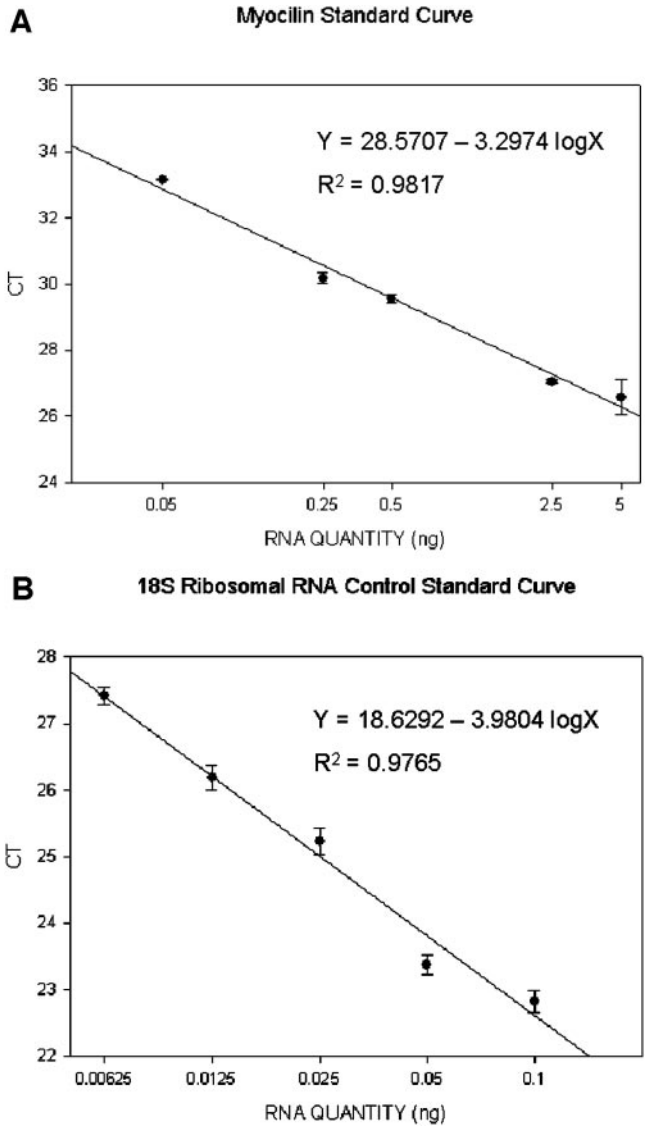

FIGURE 3. The real-time PCR standard curve for the amplification of the myocilin gene (**A**) and the 18S rRNA control gene (**B**). The equations that were generated from the data are shown above the curves.

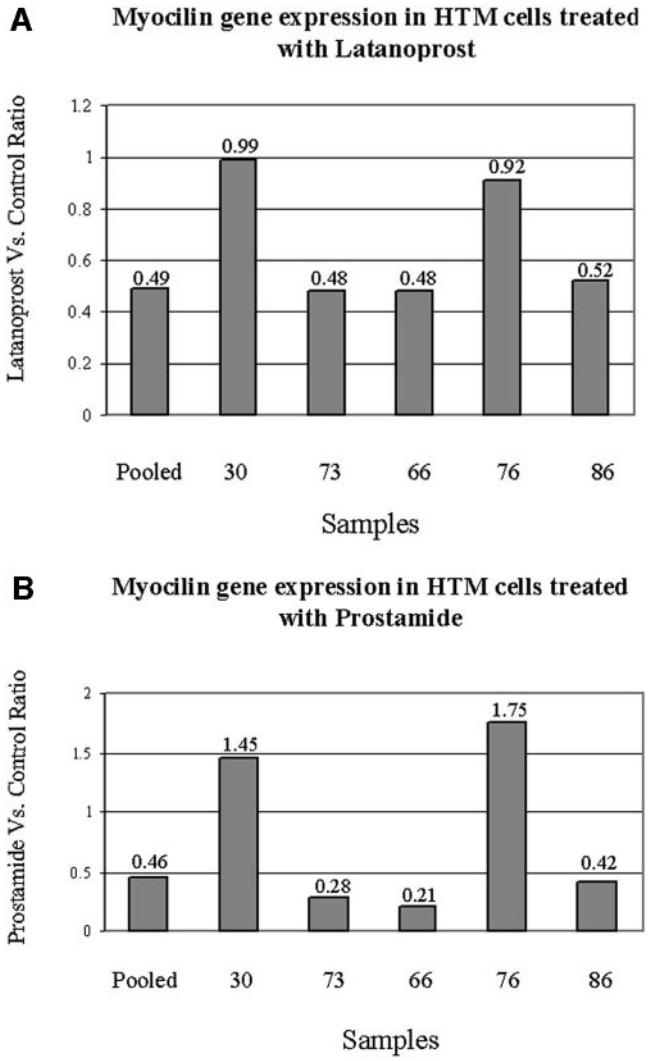

FIGURE 4. Relative quantitation by real-time PCR of myocilin gene expression in HTM drug-treated cells and HTM control cells in a pooled sample and in five individual samples.

time. These two samples had sufficient mRNA remaining to allow us to run these comparisons. There was some variation from one sample to another, but in the two individual samples as well as the pooled sample, with treatment, there were decreases in the prostaglandin receptor greater than our threshold of twofold (Fig. 6B). The changes were significant, with $P < 0.001$ for the data in the treatment groups compared with the results in the control cells in each of the assays, by Student's *t*-test.

In the HCM cells, changes in two other genes could relate directly to a possible change in outflow through the ciliary muscle (Table 3). In the case of aquaporin-1, the array indicated a decrease in expression of 6.2-fold with latanoprost treatment. With the RQ-PCR, the latanoprost treatment caused a 1.5-fold decrease, whereas the prostamide decreased expression by approximately 5.4-fold. For the other mRNA of interest in the HCM cells, versican, the array indicated a decrease of 3.3-fold in the latanoprost-treated sample. By RQ-PCR this decrease was 2.0-fold, and the prostamide-treated cells had a 3.7-fold decrease in expression of this gene. As with the aquaporin-1, no significant change of expression of versican was detected in the treated HTM cells by either the array or RQ-PCR.

However, two genes that had no changes in expression in the HCM cells detected by either the array or RQ-PCR, had very significant changes in expression in the HTM cells (Table 3). The gene that had the most change in expression was fibroleukin. The increase in the other message, insulin-like growth factor-1 (*IGF-1*), was detected by the array to be 2.8-fold with latanoprost and 4.2-fold with prostamide. Increases of 4.5-fold with latanoprost and 5.5-fold with prostamide were detected with RQ-PCR. The expressions of two additional genes, promelanosome-concentrating factor, and tumor necrosis factor superfamily 10 (*TNFSF10* or *TRAIL*), are noteworthy. Both of these genes had increased expression in the microarray and the RQ-PCR in the HTM cells, but, curiously, the RQ-PCR detected decreases in expression of these two in the HCM cells under the two treatment conditions, although the microarray indicated little change in the ciliary cells. These results were repeated several times, and although the values differed slightly, the data indicated consistently that the mRNAs were increased in HTM cells and decreased in HCM cells with both genes.

DISCUSSION

This is the first study to examine the cellular effects of both latanoprost and prostamide on HCM and HTM cells. The gene microarrays detected changes in expression of only approximately a dozen genes when HCM and HTM cells were incubated with the prostaglandin $F_{2\alpha}$ analogues, but several of these genes could have profound effects on extracellular matrix and cellular metabolism. The paucity of changes in gene expression was somewhat surprising, considering we had treated the cells with a concentration of the analogues far in excess of what would be present in a medicated eye, but with both treatments, the changes in expression within the different cell types were similar. Thus, if the mechanisms of action of the latanoprost and prostamide are different, our results could not document them. Our purpose in this study was to determine changes in metabolism of the HCM and HTM cells with continued treatment with these drugs rather than examine the acute effects of the drugs on the cells.

Two of the genes in the HCM and two in the HTM that were altered with drug treatment appear to be candidates to increase outflow. In the HCM, the decreased expression of both aquaporin-1 and versican should cause more aqueous to flow out of the eye and thus cause decreases in IOP. In the aquaporin-1–knockout mouse, IOP is decreased compared with that in a normal animal[13]; thus, the action of the prostaglandin

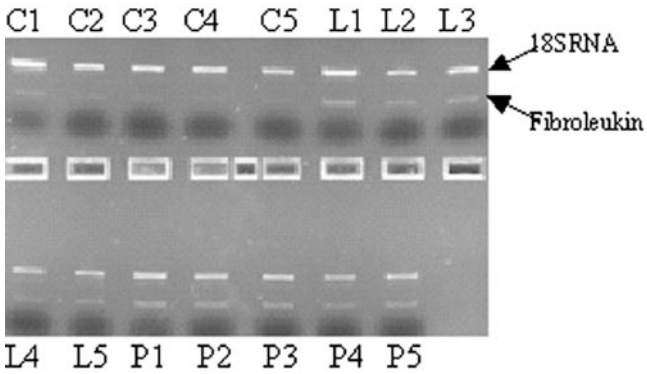

FIGURE 5. Agarose gel of the ethidium bromide–stained products of the RQ-PCR of the fibroleukin gene expression in HTM control cells and HTM cells with drug treatment. HTM control (C1–C5), HTM with latanoprost treatment (L1–L5), HTM with prostamide treatment (P1–P5).

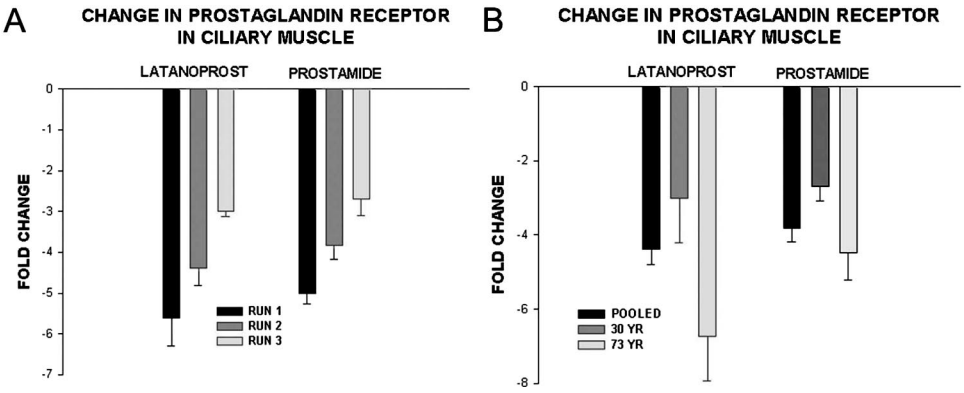

FIGURE 6. Changes in the expression of the FP receptor in HCM cells with drug treatment compared with control. (**A**) The RQ-PCR assay was run with the same DNA on three different days. The magnitude of change is the amount of decrease from the control values. Error bars indicate the standard deviation in five reactions run for each sample, compared with the normalized control value. (**B**) Changes in expression of the FP receptor in HCM cells with drug treatment. The changes in expression levels observed by RQ-PCR from DNA samples of the 30- and 73-year-old donors were compared with the change in the pooled sample marked as run 2 in (**A**). Error bars, SD.

$F_{2\alpha}$ analogues in reducing expression of this gene may also help in increasing outflow through the ciliary muscle. The increase in versican in the extracellular matrix of the trabecular meshwork with POAG suggests that this glycosaminoglycan may decrease facility.[14] If a similar obstruction to flow is present in the ciliary muscle as a result of versican content, then the reduction in expression of this gene would seem beneficial to increase the outflow as well.

IGF-1 has been reported to be present in human aqueous humor and [15] bovine trabecular meshwork cells,[16] and the receptors for this growth factor have been reported in trabecular meshwork cells.[17,18] It has also been reported that IGF-1 can increase somewhat the level of stromelysin and gelatinase B in trabecular meshwork cells. Because both the microarray and the RQ-PCR detected increases in the message for IGF-1 in the HTM cells treated with the prostaglandin analogues, it is conceivable that increases in the level of these two metalloproteinases could influence outflow through the trabecular meshwork. If this is the case, then both latanoprost and prostamide may act on the trabecular meshwork to increase facility. In addition, changes in metabolic activity are linked to this growth factor.[18] The other gene that was upregulated in the HTM that could influence extracellular matrix is fibroleukin. This protein is a serine protease that was characterized by its procoagulant properties.[19–22] The enzyme activity of this protein can cleave prothrombin to yield thrombin. Although the fibroleukin clearly is active with prothrombin as a substrate, other substrates may exist. It could be that fibroleukin is active against a component in the extracellular matrix of the trabecular meshwork and could increase outflow as a result of its protease activity. This is the first report of expression of fibroleukin in the trabecular meshwork. Further work is needed to

TABLE 3. Magnitude of Change in Gene Expression of Latanoprost and Prostamide

Gene		HCM			HTM	
		RQ-PCR	Array		RQ-PCR	Array
Aquaporin 1	C	1.00 ± 0.16	—	C	1.00 ± 0.15	—
	L	−1.57 ± 0.21	−6.2	L	1.34 ± 0.18	+1.2
	P	−5.39 ± 0.88	−1.1	P	1.26 ± 0.06	−3.35
Versican	C	1.00 ± 0.17		C	1.00 ± 0.32	—
	L	−2.03 ± 0.55	−3.3	L	1.05 ± 0.16	−1.5
	P	−3.77 ± 0.73	−1.8	P	1.07 ± 0.07	+1.2
Prostaglandin receptor	C	1.00 ± 0.04	—	C	1.00 ± 0.13	—
	L	−4.38 ± 0.43	—	L	1.09 ± 0.12	—
	P	−3.82 ± 0.36	—	P	−1.11 ± 0.06	—
IGF-1	C	1.00 ± 0.08	—	C	1.00 ± 0.20	—
	L	1.11 ± 0.08	0.0	L	4.50 ± 0.53	+2.8
	P	1.34 ± 0.30	−1.2	P	5.52 ± 0.91	+4.2
Fibroleukin	C	1.02 ± 0.13	—	C	1.00 ± 0.21	—
	L	0.71 ± 0.43	+0.8	L	2.57 ± 0.33	+7.3
	P	1.00 ± 0.24	0.0	P	6.11 ± 0.93	+15.0
Promelansome-concentrating hormone	C	1.00 ± 0.16	—	C	1.00 ± 0.09	—
	L	−2.61 ± 0.19	0.0	L	2.51 ± 0.18	+3.8
	P	−2.87 ± 0.24	−0.1	P	2.95 ± 0.41	+7.1
TRAIL	C	1.00 ± 0.16	—	C	1.00 ± 0.09	—
	L	−6.78 ± 2.13	−1.6	L	2.66 ± 0.20	+2.5
	P	−4.12 ± 0.58	−1.8	P	2.90 ± 0.61	+3.3

C, control treatment; L, latanoprost treatment; P, prostamide treatment.

investigate the role and substrates for this protease in the outflow pathway.

The increases in expression of two genes in the HTM are more difficult to interpret at the present time. The increase of TRAIL in the HTM, but its apparent decrease in the HCM, is intriguing. This protein has been studied because of its action in the immune system.[23,24] It has apoptotic activity against tumor cells that may not be present in the trabecular meshwork, although increased cell loss has been reported with glaucoma.[25] It is possible that the increased expression of the gene for TRAIL is related to the phagocytotic activity of the trabecular meshwork.

The other gene that had increased expression in the HTM but decreased in the HCM was promelanosome-concentrating hormone. This is a very complex gene that encodes messages for melanin-concentrating hormone (MCH), the neuropeptides EI and GE, an MCH gene overprinted polypeptide, and two splice variants of an antisense RNA-overlapping MCH protein.[26] These variants in rat are thought to be DNA-RNA-binding proteins[27] although these proteins have not yet been reported in humans. Complicating the picture somewhat are two variant genes for MCH on chromosome 5 (instead of chromosome 12) that appear to be active in humans, although not in rats.[28] The microarray detected the promelanosome-concentrating hormone on chromosome 12, and our oligos were designed to recognize this message rather than the mRNA of either of the variants. The increased expression of this gene initially was of interest because of the increased pigmentation in the iris and periorbital tissue in some samples treated with the prostaglandin analogues. Although this hormone has effects on pigmentation in lower vertebrates that are opposite to those of melanosome-stimulating hormone (α-MSH), in higher vertebrates, this hormone may be involved in increasing dispersion of pigment.[29,30] However, this is not the only function of MCH. This hormone is an antagonist for α-MSH in grooming behavior[31] and in the auditory sensory paradigms[32] thought to be associated with schizophrenia. In addition, MCH receptor–deficient mice are lean and hyperactive, with altered metabolism.[26] The transgenic animals that overexpress MCH are hyperphagic and tend to be obese.[33] Clearly, this hormone has significant effects on cellular metabolism that probably eclipse any effects on pigmentation.

One of the genes that may have a profound impact on long-term efficacy of the analogues is the prostaglandin FP receptor. This receptor is present in the circular muscle and collagenous connective tissues of the ciliary body.[34] It is thought that the latanoprost needs the receptor for its effect on the HCM, whereas there is some suggestion that the prostamide may not.[4] With both compounds, a reduction in expression of the receptor was observed. The long-term consequences of a reduction in the receptor may impact on the action of the drug. If this finding with cultured cells is confirmed in tissue samples, then it may be that, in certain individuals, the robustness of action of some analogues decreases with time.

Some of the changes that were detected and confirmed by real-time PCR or RQ-PCR suggest that certain genes should be more closely investigated and may influence long-term cellular homeostasis. The prostaglandin analogues, while showing effects in either HCM or HTM cells, did not show common effects in the two cell types. This suggests that the action of the prostaglandin analogues may be cell specific and may rely on other transcriptional factors present in the cell for their modes of action. The microarray data have given us insight into the actions of the prostaglandin analogues and suggest that other genes, apart from the metalloproteinase genes previously reported,[35] also contribute to increasing the outflow of aqueous humor.

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