Prostaglandin FP Agonists Alter Metalloproteinase Gene Expression in Sclera

Robert N. Weinreb,1 James D. Lindsey,1 George Marchenko,2 Natalia Marchenko,2 Mila Angert,1 and Alex Strongin2

PURPOSE. The present study was undertaken to determine whether exposure of the sclera to prostaglandin (PG)F2α or to the PGF2α analogue latanoprost acid alters mRNA for matrix metalloproteinases.

METHOD. Fifteen human eye bank eyes were studied. Circular pieces of sclera were either immediately preserved in a stabilization reagent or cultured in low-serum DMEM/F-12 medium. The cultures were treated for 24 hours with medium supplemented with PGF2α, latanoprost acid, or vehicle. Total RNA was then isolated, and the expression of mRNA for matrix metalloproteinase (MMP)-1, -2, -3, -8, -9, -10, and -12 were determined by real-time PCR. All results were normalized according to the GAPDH mRNA in each sample. Altered mRNA expression after PG treatments also was evaluated with microarrays containing 19 MMP genes and 4 tissue inhibitor of matrix metalloproteinase (TIMP) genes.

RESULTS. Real-time PCR results showed that 24 hours of exposure to 100 nM PGF2α significantly increased mRNA for MMP-1 and -9 (P < 0.06 Wilcoxon test) and that exposure to 100 nM latanoprost acid significantly increased mRNA for MMP-9 (P < 0.06 Wilcoxon test). Array analysis demonstrated increases of MMP-3 and -10 mRNA after exposure to 100 nM latanoprost and further increases after exposure to 200 nM latanoprost. The array results also showed that latanoprost induced dose-dependent increases in the expression of TIMP-1, -2, and -3 mRNA in the scleral cultures.

CONCLUSIONS. PGF2α and latanoprost acid induce coordinated alterations of MMP gene transcription in scleral organ cultures. These results indicate that PGs can directly trigger MMP gene transcription changes within the sclera. These changes support a role for increased MMPs in the enhancement of uveoscleral outflow that occurs after topical treatment with latanoprost. (Invest Ophthalmol Vis Sci. 2004;45:4568–4577) DOI: 10.1167/iovs.04-0411

The reduction of intraocular pressure after topical treatment of monkey eyes with prostaglandin (PG)F2α reflects increased uveoscleral outflow facility1,2 and is associated with decreased fibrillar collagen3 and increased matrix metallopro-

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MMPs, also may contribute to the scleral response to PGs, as they can degrade nicked fibrillar collagens or mediate pro-MMP activation.6,17 Moreover, the activity of MMPs in the sclera may be regulated by the production of tissue inhibitors of matrix metalloproteinases (TIMPs).18,19 However, it is unknown whether similar responses by these MMP mRNAs also occur in PG-treated sclera.

In addition to MMP-1, -2, and -3, several other MMPs may contribute to the reduction of extracellular matrix in the sclera. For example, MMP-8 and -13 (collagenase-2 and -3) can cleave intact fibrillar collagens similarly to MMP-1 (collagenase-1).10–12 Likewise, MMP-9 (gelatinase B) can hydrolyze fibrillar collagen fragments similarly to MMP-2 (gelatinase A).13,14 MMP-10 (stromelysin-2) can mimic the ability of MMP-3 (stromelysin-1) to activate certain pro-MMPs.15,16 Several other MMP types, such as the elastase MMP-12 or the membrane-type MMPs, also may contribute to the scleral response to PGs, as they can degrade nicked fibrillar collagens or mediate pro-MMP activation.6,17 Moreover, the activity of MMPs in the sclera may be regulated by the production of tissue inhibitors of matrix metalloproteinases (TIMPs).18,19 However, it is unknown whether PG treatment induces changes in scleral mRNA of these additional MMPs or TIMPs. Thus, the present study was undertaken to survey the effect of PGF2α and latanoprost acid on MMP and TIMP gene transcription using real-time PCR and microarray analysis of gene expression.

METHODS

Fifteen postmortem human eyes were obtained from the San Diego Eye Bank and the University of California San Diego Body Donation Program (Table 1). Donors had no history of glaucoma or other ocular diseases. Eyes were enucleated within 5 hours after death and immediately preserved in a moist chamber at 4°C. Only intact eyes were included, and any eye showing scleral damage or posterior staphyloma was excluded from study. Within 24 hours after preservation, the sclera was dissected and placed into organ culture, as previously described.9 Briefly, the eyes were first incubated for 30 minutes in Hanks’ buffered saline solution (HBSS) containing 50 U/mL penicillin and 50 U/mL streptomycin. Next, residual extraocular muscles and orbital connective tissues were removed. The sclera then was dissected into four pieces. Care was taken to avoid including the long ciliary nerve and artery, insertion points of muscles, or vortex veins in each center area. Uveal tissue and the retina were gently removed from the sclera with a cotton-tipped applicator. Scleral pieces were placed into 12-well plates containing DMEM/F-12 supplemented with 1% FCS

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and 1 ng/mL human recombinant FGF-2 (17 kDa; Invitrogen-Gibco, Grand Island, NY). Because serum contains various stimulating factors of MMP synthesis, a low concentration was used to minimize the nonspecific increase of MMP. The explants were incubated at 37°C in a humidified atmosphere of 95% air /179/5% CO2.

**Prostaglandin Treatment**

To investigate the effect of PGF2α or latanoprost acid, the biologically active component of latanoprost, on scleral MMP mRNAs, we changed the culture medium to fresh medium supplemented with the respective PG (Cayman Chemical Co., Ann Arbor, MI). Tested concentrations included 100 and 200 nM, since the peak concentration in human aqueous humor after topical application of a clinical dose is 100 nM (Sjöquist B, et al. *IOVS* 1997;38:ARVO Abstract 1148). The cultures were treated with PGs for 24 hours because a previous study found moderately increased MMPs in the medium of scleral cultures exposed to PGs for 24 hours,9 and because the increased MMP mRNA in ciliary muscle cells exposed to PGs was generally maximum by 24 hours after PG treatment.20

**RNA Isolation and Reverse Transcription Reaction**

Total RNA was harvested from the sclera organ cultures, according to a modification of the protocol for RNA isolation (RNeasy; QiaGen, Valencia, CA). Briefly, scleral pieces were homogenized in ice-chilled guanidine thiocyanate solution (buffer RLT) with a rotor-stator homogenizer (Polytron PT10/35; Brinkman Instruments, Westbury, NY). The homogenate was digested with 20 milli-Anson units (mAU)/mL proteinase K at 55°C for 20 minutes and centrifuged. The supernatant was diluted 1:1 with 70% ethanol, applied to a silica-gel membrane column (RNeasy minicolumn; QiaGen), and washed with buffer RW1. To eliminate residual genomic DNA, the column was incubated with 27 Kunitz units of DNase (QiaGen) at 37°C for 15 minutes. After washing with buffer RW1 and then buffer RPE, the column was eluted with 30 mL RNase-free water. RNA yield, purity, and quality were evaluated by spectrophotometry at 260 and 280 nm.

To assess the accuracy of the procedures, triplicate aliquots of total RNA were used to produce cDNA in parallel reactions. First-strand cDNA was synthesized with RNase H reverse transcriptase purified from *Escherichia coli* containing the *pol* gene of Moloney murine leukemia virus.

**Table 1. Donor Information**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Cause of Death</th>
<th>Death to Enucleation*</th>
<th>Death to Utilization †</th>
<th>Eye</th>
<th>Ocular History</th>
<th>Eye Label</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>M</td>
<td>Ischemic bowel disease</td>
<td>2:30</td>
<td>—</td>
<td>OD</td>
<td>Lens implant</td>
<td>1R</td>
</tr>
<tr>
<td>2</td>
<td>86</td>
<td>F</td>
<td>Cerebral vascular accident</td>
<td>7:45</td>
<td>—</td>
<td>OD</td>
<td>—</td>
<td>2R</td>
</tr>
<tr>
<td>3</td>
<td>76</td>
<td>F</td>
<td>Lung cancer</td>
<td>4:05</td>
<td>28:05</td>
<td>OD</td>
<td>—</td>
<td>3R</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>M</td>
<td>Lung cancer</td>
<td>3:20</td>
<td>34:55</td>
<td>OD</td>
<td>—</td>
<td>4R</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>M</td>
<td>Chronic pulmonary obstruction</td>
<td>4:14</td>
<td>14:34</td>
<td>OD</td>
<td>—</td>
<td>5R</td>
</tr>
<tr>
<td>6</td>
<td>77</td>
<td>M</td>
<td>Cardiac arrest</td>
<td>28:20†</td>
<td>29:35</td>
<td>OD</td>
<td>—</td>
<td>6R</td>
</tr>
<tr>
<td>7</td>
<td>62</td>
<td>M</td>
<td>Pneumonia</td>
<td>4:15</td>
<td>11:50</td>
<td>OD</td>
<td>—</td>
<td>7R</td>
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<tr>
<td>8</td>
<td>88</td>
<td>F</td>
<td>Coronary artery disease</td>
<td>9:38‡</td>
<td>10:43</td>
<td>NR</td>
<td>—</td>
<td>8</td>
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<tr>
<td>9</td>
<td>76</td>
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<td>Lung cancer</td>
<td>4:05</td>
<td>28:30</td>
<td>OD</td>
<td>—</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>81</td>
<td>F</td>
<td>Cardiorespiratory arrest</td>
<td>18:35‡</td>
<td>19:50</td>
<td>NR</td>
<td>—</td>
<td>10</td>
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</tbody>
</table>

* Death to enucleation/refrigeration time (hours:minutes).
† Death to utilization time (generation of scleral organ cultures).
‡ Bodies from this source refrigerated within 4 hours after death.

**Table 2. Sequences of the Primers for Real-Time PCR**

<table>
<thead>
<tr>
<th>MMP-1 (interstitial collagenase)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACT GAA CCT GAC GGT ACA GGC TGA AAG TGA CTG GGA AA</td>
<td>CAC ATC AGG CAC TCC ACA TC</td>
</tr>
<tr>
<td>MMP-2 (gelatinase A)</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td></td>
<td>ACT GAA CCT GAC GGT ACA AGG ACT AGG ACC GGG ACA AG</td>
<td>GTC ACC ACC AGT GGA CAT</td>
</tr>
<tr>
<td>MMP-3 (stromelysin-1)</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td></td>
<td>ACT GAA CCT GAC GGT ACA AAC CTG TCC TCT CAG AAC CT</td>
<td>CAG CAT CAA AGG ACA AAG CA</td>
</tr>
<tr>
<td>MMP-8 (collagenase-2)</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td></td>
<td>ACT GAA CCT GAC GGT ACA TCA GGT GCC TTT GCC TTTCCA GGA AT</td>
<td>GGT CGA CTG AAG ACA TGG AAG AA</td>
</tr>
<tr>
<td>MMP-9 (gelatinase B)</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td></td>
<td>ACT GAA CCT GAC GGT ACA GGC GGT CAT GTA CCC TAT GT</td>
<td>GCC ATT CAC GTC GTC CTG TT</td>
</tr>
<tr>
<td>MMP-10 (stromelysin-2)</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td></td>
<td>ACT GAA CCT GAC GGT ACA GAG GCA GCA AAT</td>
<td>TTC CTG CTC AGA AAC AGG TGC AT</td>
</tr>
<tr>
<td>MMP-12 (elastase)</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td></td>
<td>ACT GAA CCT GAC GGT ACA GAG ATC</td>
<td>TTC GTC CTC ACG GAA ATG TG</td>
</tr>
</tbody>
</table>
leukemia virus (Superscript II; Invitrogen-Gibco). The 20-μL reaction volume contained 1 to 5 μg total RNA, 0.5 μg oligo (dT), 50 mM Tris (pH 8.3 at room temperature), 75 mM KCl, 3 mM MgCl₂, 0.01 M dithiothreitol, 0.5 mM dNTPs, and 200 U reverse transcriptase. The reaction mixture was incubated at 70°C for 15 minutes.

**Real-Time PCR**

MMP-1 (collagenase 1), -2 (gelatinase A), -3 (stomelysin 1), and -9 (gelatinase B) mRNAs were investigated in this study because these gene products had been shown to be increased in ciliary muscle cells exposed to latanoprost. MMP-8 (collagenase 2), -10 (stomelysin 2), and -12 (elastase) mRNAs were studied because pilot studies with the microarray analysis described later in the present article had indicated possible alterations in the amounts of these gene products after exposure to PGF₂α or latanoprost acid. The expression of these gene products was measured using a modification of the Taqman real-time PCR method. With this method of real-time PCR (Amplitifuor; InterGen Co., Purchase, NY), a hairpin configuration oligonucleotide containing a fluorophore and quencher becomes incorporated into the specific PCR product produced with each cycle. The amount of fluorescence present in each reaction was monitored during each PCR cycle. The signal arose from separation of a quencher and a fluorophore that occurs by opening up the hairpin. With subsequent cycles, the signal rises and then plateaus. A short oligonucleotide sequence, 5’-ACTGAAACCTGACGGTACA-3’ was added to the 5’ end of the forward primer. This sequence specifically binds a hairpin configuration oligonucleotide containing the quenched fluorophore that also is present in the reaction mixture (Amplifluor Uniprimer; InterGen Co.). Each determination of the specific amount of MMP mRNA was normalized according to GAPDH mRNA also present in each sample. The sequence of these primers is shown in Table 2.

**Specificity**

The specificity of each PCR assay was confirmed by electrophoresis of the reaction products using cDNA generated from OCM-1 cells as well as human sclera. For each assay, 15 μL of the reaction mixture containing reaction products from amplified reaction plates was separated by electrophoresis in a 4% agarose gel (NuSeive; Cambrex Corp., East Rutherford, NJ). The running buffer contained 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA (pH 8.3; TBE buffer). Each gel included 100- and 25-bp ladder standards (Promega, Madison, WI). No-template controls contained primer probes and PCR reagents, but did not have cDNA template added. A second control reaction also contained primer probes and PCR reagents, but purified RNA from scleral cultures had not been reacted with reverse transcriptase was used as the template. Experimental assays contained probes, primer, PCR reagents, and scleral culture cDNA. The no-template controls were used to evaluate the possibility of nonspecific amplification of primer or probe sequences. The gels were developed with ethidium bromide and photographed on a light box with 360-nm excitation (Transilluminator 4000; Stratagene, La Jolla, CA). The purpose of these experiments was to determine whether the size of the PCR product was the same as predicted from the sequence of the amplicon and whether nonspecific amplification products were absent. The linearity of four of the real-time PCR assays has been confirmed with serial dilutions of custom oligonucleotide standards.

As a further control for the specificity of the assays, PCR reaction products from several assays were analyzed by sequencing. After real-time PCR, the amplified DNA was purified from the other reaction mixture components by binding to a silica gel membrane column in a high-salt buffer and then eluting in a low-salt buffer (QiQuick; Qiagen). The purified DNA was replicated by PCR using fluorescent dye terminator dyeoxyxucleotides, polymerase FS (AmpliTaq FS; BigDye Terminator, ver. 2.0, Applied Biosystems, Foster City, CA), and the reverse primer from the real-time PCR reaction. The reaction products were analyzed by gel electrophoresis in an automated DNA sequencer (model 3100; Applied Biosystems). The fluorescence fragments were identified as they passed through a laser beam detector positioned over the gel. Electrophorograms from the sequencer were then analyzed on computer (EditView, ver. 1.0.1; Applied Biosystems).

**MMP-TIMP Microarray**

Gene microarray membranes for matrix metalloproteinases (GEArray; SuperArray, Inc., Gaithersburg, MD) were used according to hybridization procedures described by the manufacturer. Total RNA was isolated, and cDNA was generated as described for RT-PCR, with 4 μM [α-33P-dCTP] (10 μCi/μL; 3000 Ci/mmol, Amersham Pharmacia BioTech, Piscataway, NJ). The array filters were prehybridized with heat-denatured salmon sperm DNA for 2 hours and then hybridized with the labeled cDNA at 60°C for 20 hours. The filters were washed twice with 2× SSC buffer containing 1% sodium dodecyl sulfate (SDS) and then twice with 0.1× SSC and 1% SDS. The filters were then exposed to x-ray film (X-O-Mat; Eastman Kodak, Rochester, NY) for 24 to 72 hours. The density of the spots on the film was measured with an imaging densitometer.

**Statistical Analysis**

The mean and standard deviation of the real-time PCR measurements were determined. Because the results were not normally distributed, comparison of MMP induction in vehicle- and PG-treated cultures was made using the Wilcoxon paired-sample test. In the Wilcoxon test, the discrete nature of the distribution of possible values of W means that
one cannot always obtain probabilities ($P$) precisely at traditional levels, such as 5%. For example, when $n = 6$, as was the case in several of the experimental groups in the present study, the critical $W$ of 21 and 19 yield $P$ of 0.032 and 0.062, respectively. Thus, for the sake of uniformity, the results were considered to be significant when $P < 0.06$ for all experiments in the present study.

### RESULTS

#### Specificity Real-Time PCR Assays

To ensure that the origin of the signal reflected amplification of the target cDNA, reaction mixtures from a completed real-time PCR run were separated by electrophoresis and then stained with ethidium bromide. As shown in Figure 1A, each lane that contained the complete reaction mixture generated one band that corresponded in size to the expected molecular size of the PCR product for MMP-1, -2, -3, and -9. The results of for MMP-8, -10, and -12 were similar (not shown). To assess whether the nonspecific reaction products were generated, reactions run without template were compared with complete reactions containing template (Fig. 1B). The absence of bands in the reactions without template is consistent with specific amplification of the targeted MMP cDNA by the real-time PCR assays.

To determine whether the purified RNA contained genomic DNA contamination, a PCR reaction that contained purified RNA from scleral cultures that had not been reacted with reverse transcriptase was used as the template as well as the primer probes and PCR reagents. No bands were present after electrophoresis of the reaction products (not shown), indicating no contamination of the RNA by genomic DNA.

#### Real-Time PCR Analysis of MMP mRNA in Untreated Cultures

The mRNA for MMP-1, -2, -3, -8, -9, -10, and -12 was detected by real-time PCR in cultures from eyes freshly obtained from the eye bank. The magnitude of expression ranged from 10 times less than the expression of GAPDH for MMP-3 to 10,000 times less than GAPDH expression for MMP-9 and -12 (Fig. 2). Maintenance in culture for 24 hours increased the expression of all these MMP mRNAs except MMP-2. In most cases, expression was increased 3- to 30-fold. In contrast, MMP-12 increased an average of 300-fold in the cultures, whereas mean MMP-2 mRNA expression in the cultured sclera decreased to approximately one third of the expression in fresh sclera.

#### Real-Time PCR Analysis of MMP mRNA in PG-Treated Cultures

Compared with vehicle-treated cultures, increased MMP-1 mRNA was observed in 100 nM PGF$_{2x}$-treated cultures in five (83%) of six donor eyes (Fig. 3A). The mean increase was 1.9-fold, and was significant by the Wilcoxon paired-sample test ($P < 0.06$). Increased MMP-9 mRNA was observed in PGF$_{2x}$-treated cultures from six (86%) of seven donor eyes. Mean MMP-9 mRNA increased by 4.2-fold, which was significant by the Wilcoxon test ($P < 0.06$). Changes in MMP-2, -3, -8, -10, and -12 mRNA expression in the treated cultures were variable and insignificant by the Wilcoxon test.

Human scleral organ cultures exposed to 100 nM latanoprost acid for 24-hours contained increased expression of MMP-9 mRNA in cultures from seven (78%) of nine donor eyes (Fig. 3B). Mean MMP-9 mRNA increased by 3.2-fold, which was significant by the Wilcoxon paired-sample test ($P < 0.06$). Although increased MMP-1 mRNA expression after latanoprost acid treatment was observed in cultures from 9 (69%) of 13 donor eyes examined, this change was nonsignificant by the Wilcoxon test. Also nonsignificant were the responses of mRNAs of MMP-2, -3, -8, -10, and -12.

#### Confirmation of PCR Products

For further confirmation of the specificity of the protocol for the real-time PCR reactions of MMP-1, -2, and -9, the amplicons...
in the reaction mixtures after completion of these assays were sequenced with a dye termination protocol. A perfect match was obtained for 56 of the 76 bases in the amplicon for MMP-1. For MMP-2, a perfect match was obtained for 28 of 67 bases. For MMP-9, a perfect match was obtained for 42 of 62 bases. In each case, these results indicate that the signals measured in the real-time PCR reactions for these MMPs specifically measured the correct mRNA only. These results support the specificity of the real-time PCR assays used in the investigation.

MMP-TIMP Array Analysis of Fresh and Cultured Sclera

Total RNA was isolated from the sclera tissue of eyes 3R, 8, 9, and 10 and arrayed on the MMP-TIMP nylon arrays. The samples included fresh sclera, sclera incubated in medium alone (vehicle), sclera incubated with 100 nM latanoprost acid, and sclera incubated with 200 nM latanoprost (illustrated in Fig. 4). The most intense signals in the fresh sclera arrays were for TIMP-2 and -3 mRNA (Fig. 5). TIMP-1 mRNA expression in these cultures was much lower. No expression of TIMP-4 was detected. Among MMPs, the highest levels were detected for MMP-10, -2, and MT6-MMP mRNA. Minor expression was detected of MMP-3, -8, -19, -26, and MT1-MMP mRNAs. Expression of mRNA for some MMPs, such as MMP-9, was undetectable.

Incubation in culture medium for 24 hours substantially increased expression of MMP-3 (15–20-fold), -10 (10–15-fold), and -12 (Figs. 4, 5). The expression of MMP-8 in the culture from eye 8 increased approximately 12-fold. Small increases also were observed in the expression of MMP-19 and -26. Among the TIMPs, the expression of TIMP-1 was increased, whereas the expression of TIMP-2 and -3 were reduced.

MMP-TIMP Array Analysis of Sclera Cultures Exposed to Latanoprost

MMP-TIMP array analysis of RNA from cultures exposed to 100 nM latanoprost acid, 200 nM latanoprost acid, or vehicle revealed latanoprost-associated increases in the expression of mRNAs for MMP-3, -10, and MT1-MMP (Fig. 6). Exposure to latanoprost acid also increased the expression of mRNAs for TIMP-1, -2, and -3. In the case of eye 8, expression of MMP-8 was not detected in either of the latanoprost-treated cultures.

Comparison of Real-Time PCR and Array Results

Comparison of the results for each of the MMP mRNA types analyzed by both procedures is summarized in Table 3. For MMP-1, the amount of mRNA in the control cultures was 0.02% of the amount of GAPDH mRNA. Although this was sufficient for the real-time PCR assays, it was insufficient to provide a
that were hybridized with labeled cDNA from sclera organ cultures control 1 (plasmid DNA); C2, control 2 (β-actin); and C3, control 3 (GAPDH). Note that MMP-14, -15, -16, -17, -24, and -25 are also known as MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP, MT5-MMP, and MT6-MMP, respectively.

strong signal in the array assays. By real-time PCR, MMP-1 mRNA in PGF$_{2\alpha}$-treated cultures from 66% of the donors had increases exceeding 1.5-fold and the response overall was significantly increased by either PG treatment. Thus, it appears reasonable to conclude that PGF$_{2\alpha}$ treatment generally increased MMP-1 transcription. Although latanoprost treatment also resulted in increased MMP-1 in cultures from twice as many donor eyes as responded with no change or decreases, the response was smaller and overall was not significant by the Wilcoxon test. To indicate this difference in Table 3, the conclusion “Increased” is followed by “P/(L)”. Similar consideration was given to each of the other MMPs that also were analyzed with both methods.

In the case of MMP-9, the amount of signal was too low to be seen by the array analysis. However, the more sensitive real-time PCR method consistently found increased MMP-9 mRNA after treatment with either FP agonist. With MMP-3 and -10, the PCR results were equivocal, but the array results consistently supported increased mRNA expression. Both the PCR and the array results indicate that MMP-2, -8, and -12 mRNAs were not significantly increased by either PG treatment.

**DISCUSSION**

Exposure of isolated human sclera to PGF$_{2\alpha}$ or latanoprost increased mRNA for several MMPs and TIMPs. These responses typically were less than fivefold in magnitude. The two methods used in the present study have complementary strengths and limitations that, when considered together, facilitate interpretation of the results. Hence, the strengths and limitations of these methods will be compared first, then the limitations of the experimental material studied in the this investigation will be considered, and finally conclusions will be developed based on these considerations and the results of the assays.

The primary strength of the real-time PCR method is its wide dynamic range. This allows comparison of results that may be 1000-fold different in magnitude with good accuracy. For example, we have found that regression analysis of a serially diluted template using computer-aided analysis of the real-time PCR results typically yields mean threshold cycle ($C_T$) values within 0.2-fold of the regression line for the serial dilution. Standard deviations of triplicate determinations in this study typically were <0.3-fold. Normalization of each result in the present study according to GAPDH mRNA expression provides correction for specimen pipetting variations. Thus, differences of at least 1.5-fold are likely to be meaningful.

In the present study, the main limitation of the real-time PCR method is that the small amount of mRNA available from each sclera organ culture was sufficient to assess only four to six different MMP mRNA types. Hence, the list of contributing donors differs for each of the various MMPs evaluated by this method.

In contrast to real-time PCR, each microarray analysis allowed simultaneous evaluation of all mRNA types represented on the array. Also, this technique has excellent linearity with strong signals. However, its dynamic range spans only approximately 100-fold and it is less sensitive than real-time PCR. To maximize sensitivity, the film exposure time was lengthened in the present study as much as possible without loss of acceptable signal-to-noise ratios. Nevertheless, changes in the smaller amounts of MMP-1 and -9 mRNA which were readily measured by real-time PCR reactions, were not measurable in the microarray assays. Because of the superior linearity of the microarray results when the signal was strong, it appears appropriate to give more weight to the array results for relatively abundant mRNA species including MMPs-3, -8, -10, and -12, as well as TIMPs-1, -2, and -3. As strong signals were not present for MMP-1, -2, or -9 mRNAs in the arrays, only the real-time PCR results could be considered. Hence, the real-time PCR assays for MMPs-1, -2, and -9 were further validated by confirming the sequences of these PCR reaction products.

Exposure of scleral cultures to PGF$_{2\alpha}$ or latanoprost increased MMP-1 mRNA. Prior investigations demonstrated increased scleral MMP-1 immunoreactivity in monkey eyes after topical PGF$_{2\alpha}$-isopropyl ester treatment and increased MMP-1 concentration in the medium of human scleral cultures exposed to PGF$_{2\alpha}$ or latanoprost acid. Hence, the increased MMP-1 protein probably was synthesized locally within the sclera. Moreover, this increased biosynthesis reflected increased transcription of the gene for MMP-1.

Changes in MMP-2 mRNA in scleral cultures treated with PGF$_{2\alpha}$ or latanoprost were not significant by the Wilcoxon test. This result is similar to the previous observation of no change in MMP-2 mRNA in human ciliary smooth muscle cell cultures exposed to latanoprost. However, increased MMP-2 immunoreactivity in the sclera of monkey eyes was observed after topical PGF$_{2\alpha}$-isopropyl ester treatment. In addition, MMP-2 concentration was increased in the medium of human scleral cultures exposed to PGF$_{2\alpha}$ or latanoprost acid. Stimulated increases in MMP-2 secretion that were not accompanied by increases in MMP-2 mRNA have been found in macrophages and vascular tumor cells. In the latter case, this secreted MMP-2 originated from intracellular stores. Further experiments are needed to determine whether the response of scleral fibroblasts to PG treatment is similar to that of these other cell types. Another possibility is that MMP-2 mRNA was increased either earlier or later than the 24-hour time point examined in the present study.
The amount of MMP-3 mRNA in the cultures was >30-fold greater than MMP-1 or -2 mRNAs. This was sufficient to produce a readily measured signal in the microarray assays. Though the real-time PCR results for this MMP were variable, the microarray results consistently showed a dose-dependent increase in MMP-3 mRNA after latanoprost treatment. Thus, the previously observed increase in scleral MMP-3 immunoreactivity within monkey sclera after topical PGF2α-isopropyl ester treatment probably reflected increased local biosynthesis. This conclusion is supported by the increased MMP-3 concentration found in the medium of human scleral organ cultures exposed to 100 nM latanoprost acid.

The amount of MMP-8 mRNA in the control cultures generally was similar to MMP-1 and -2 mRNA (i.e., 0.01% of GAPDH mRNA). Although this MMP was first identified in neutrophils, the scleral organ cultures were unlikely to contain many neutrophils. MMP-8 also has been found in several other tissue types. Hence, further investigations are needed to confirm the identity of the cell type expressing MMP-8 in sclera. In most of the cultures examined by real-time PCR, treatment with latanoprost reduced expression of this mRNA. Mixed responses were observed in the array experiments, as well, making it unlikely that MMP-8 contributed to the scleral response to PG treatments.

Expression of MMP-9 mRNA was 20 times less than that of MMP-1 or -2 mRNA and was the least abundant of the mRNA types analyzed in the present investigation. That this MMP was detected by real-time PCR and not by microarray analysis is consistent with this low abundance. Exposure to either PGF2α or latanoprost increased MMP-9 mRNA in cultures from most of the donors. This increase averaged approximately fourfold in magnitude and was significant for both agonists, by the Wilcoxon test. The increase in scleral MMP-9 mRNA appears to be similar to the increased MMP-9 in the culture medium of human ciliary smooth muscle cells exposed to PGF2α or latanoprost acid. Further experiments analyzing the concentration of MMP-9 protein in the medium of treated scleral cultures are needed to determine whether this increase in MMP-9 mRNA leads to increased MMP-9 protein biosynthesis and release. It is
also important to assess whether the amount released contributes significantly to scleral extracellular matrix remodeling.

Although the relative amounts of MMP-10 and -12 mRNA in the control cultures were similar to those of MMP-1 and -2, the signals for these mRNAs in the array assays were strong enough for quantitative comparisons. Increased MMP-10 expression after 100 nM latanoprost was further increased when the treatment dose was doubled. In contrast, MMP-12 mRNA typically was either reduced or unchanged by treatment with either agonist. Similar to MMP-9, further experiments analyzing the concentration of MMP-10 protein in the medium of treated scleral cultures are needed to determine whether this increase in MMP-10 mRNA leads to increased MMP-10 protein biosynthesis and release.

The induction of TIMP-1, -2, and -3 in the PG-treated sclera cultures suggests that the influence of increased MMPs is regulated in the extracellular spaces by these increased TIMPs. However, it is possible that the balance between the expression of certain MMPs and TIMPs is altered by the PG treatments. For example, the increases in MMP-3 and -10 shown in Figure 6 may exceed the increases in TIMP expression. In addition, a previous study found that, after the addition of latanoprost to human ciliary muscle cultures, the induction of MMP mRNAs preceded the induction of TIMP mRNAs. If this resulted in a delay in the biosynthesis of the TIMP proteins, the delay could promote MMP-mediated collagen degradation for a limited time.

The present results indicate that the MMP mRNA responses to 100 nM PGF2α/H9251 are similar to the MMP mRNA responses to 100 nM latanoprost acid. This concentration was chosen because it is the same as the peak concentration of latanoprost acid in human aqueous humor after topical application of the clinical dose of latanoprost (Sjöquist B, et al. IOVS 1997;38: ARVO Abstract 1148). Moreover, it is the same as the EC50 for the induction of phosphoinositide turnover (a measure of receptor activation) by PGF2α/H9251 or latanoprost acid in human ciliary muscle cells. Although PGF2α can activate other PG receptors besides the FP receptor, experimental studies have shown that the activation potency is >50 times less than at the FP receptor. Thus, the similar MMP and TIMP mRNA responses with these agonists are consistent with similar specific activation of the FP receptor.
### Table 3. Comparison of PCR and Array Results

<table>
<thead>
<tr>
<th>Array Analysis</th>
<th>Real-Time PCR</th>
<th>Conclusion (mRNA)</th>
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<tr>
<td>Primary Dose–Response</td>
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<td>Control Signal</td>
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<td>PCR results</td>
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<td>Array results</td>
<td>Array results</td>
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<td></td>
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<td>Control Signal¶</td>
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<td>Mixed</td>
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| MMP-1 | 0.0100 | 0.020 | 4/2/0 | 9/0/4 | 66 | 38 | 33 | 31 |
| MMP-2 | 0.1000 | 0.020 | 5/0/3 | 6/2/3 | 50 | 45 | 38 | 27 |
| MMP-8 | 0.0008 | 0.010 | 3/1/1 | 3/0/6 | 33 | 22 | 33 | 22 |
| MMP-9 | 0.0002 | 0.001 | 6/0/1 | 8/0/1 | 86 | 77 | 86 | 44 |
| MMP-10 | 0.0007 | 0.010 | 3/0/2 | 4/0/6 | 40 | 20 | 20 | 10 |

† Number of donors in which the cultures had increased mRNA expression, no change of expression, or decreased mRNA expression was greater than 1.5-fold. ‡ Percentage of donors in which increased mRNA expression was greater than twofold.

In conclusion, this study has shown that coordinated increases in the mRNA for certain MMPs and TIMPs occurs in scleral tissue exposed to PGF<sub>2α</sub> or latanoprost. Overall, these results are consistent with a shift of the metabolic profile toward reduced intrascleral extracellular matrix after PG treatment. Further investigations will be helpful in clarifying the relationships among MMP gene expression changes and extracellular MMP activities within sclera, as well as the potential link between variable gene inductions and variable IOP responses.

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### References


