Latanoprost Stimulates Secretion of Matrix Metalloproteinases in Tenon Fibroblasts Both In Vitro and In Vivo

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PURPOSE. To investigate the presence and the possible role of different matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in Tenon capsule fibroblasts. These enzymes are essential for the control of tissue remodeling in the context of wound repair. This aspect is important to further the understanding of and possibly to influence the scarring process of filtering blebs after glaucoma surgery.

METHODS. Untreated and latanoprost-treated human Tenon fibroblasts were examined for the presence of MMPs and TIMPs on the mRNA and protein levels. Assays performed included RT-PCR, real-time RT-PCR, immunocytochemistry, Western blot analysis, flow cytometry, and zymography. To investigate the changes in vivo, conjunctival specimens of rabbits treated with latanoprost eye drops were examined by immunohistochemistry.

RESULTS. In all assays, both MMP-3 and TIMP-2 were detected. With the real-time RT-PCR technique, MMP-1, -2, -3, -7, -9, and -14 and TIMP-1 and -2 were detected. An upregulation of MMP-3 and TIMP-2 after latanoprost treatment of the fibroblasts was shown and found to occur on the mRNA and the protein levels. The upregulation of MMP-3 and TIMP-2 was confirmed in vivo.

CONCLUSIONS. Tenon fibroblasts contain the ability on the mRNA level to synthesize all enzymes of the MMP and TIMP family that are related to remodeling of the extracellular matrix. The levels of MMP-3 and TIMP-2 increase after treatment with latanoprost. Tenon fibroblasts may be the target cells for attempts to influence the tissue levels of MMPs and TIMPs in the context of conjunctival wound healing after glaucoma surgery. (Invest Ophthalmol Vis Sci. 2003;44:5182–5188) DOI: 10.1167/iovs.02-0462

To date, trabeculectomy is the most frequently performed surgical procedure used in the treatment of glaucoma. Many studies have sought to further the understanding of the underlying mechanisms involved in the wound-healing process after glaucoma filtration surgery. Gains made in such studies may be useful in promoting, delaying, or inhibiting the formation of scar tissue, which frequently seals the surgically created fistula. Fibroblasts, which play an essential role in wound healing in other locations of the body, have been identified as the main cell type that produces the new tissue surrounding the scleral flap.

In the context of glaucoma surgery, the period before surgery seems to be of importance, because most patients receive topical antiglaucoma therapy before surgery—many for several years. Clinical studies have noted that patients with a history of such preoperative topical therapy have a less favorable long-term outcome of fistulizing surgical procedures. Histopathologic examinations of conjunctival specimens from such patients have revealed an increase mostly of inflammatory cells, whereas the extracellular matrix was only rarely examined. Recently, it was found that long-term topical therapy with timolol leads to a marked increase of extracellular matrix. This accumulation of extracellular matrix may be the histopathological correlate of the clinically observed reduced success of surgical procedures. The extracellular matrix is synthesized and remodeled by the tissue fibroblasts.

It has been shown that latanoprost may reduce the amount of extracellular matrix production by Tenon’s capsule fibroblasts. This effect may be controlled by metalloproteinases (MMPs) and their natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). For this reason, we decided to investigate the expression of MMP and TIMP in human Tenon fibroblasts in more detail and the effect of latanoprost on the levels of these enzymes. Ocular fibroblasts in tissue culture provide a useful in vitro model as well as a logical first step in studying the effects of new substances on ocular wound healing. In a second step, we treated rabbits with latanoprost and examined conjunctival specimens to attempt to demonstrate that the results obtained in experiments in vitro hold true in vivo as well.

MATERIALS AND METHODS

Cell Culture

Human subconjunctival Tenon fibroblasts were prepared from specimens of patients undergoing routine cataract surgery. The patients had no history of systemic or conjunctival diseases and did not take any topical ocular medications. Informed consent was obtained. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 1000 mg/L glucose (Life Technologies, Karlsruhe, Germany) with addition of 10% fetal calf serum (FCS) as previously described. Fourth-passage cells were used for most experiments.

Control cells and cells treated with latanoprost 0.5 μg/mL were handled similarly. For all but the zymography assay, cells were not serum starved. Treated cells were exposed to latanoprost at hours 0, 24, and 48. The control and treated cells were harvested at a time point of 72 hours.

For the real-time RT-PCR assay for MMP-3 and TIMP-2, cells were treated at hour 0 and harvested at 24 hours for the 1-day treatment with latanoprost. For the 2-day treatment with latanoprost, cells were treated at hours 0 and 24 and harvested at 48 hours. For the 3-day treatment, cells were exposed to latanoprost at 0, 24, and 48 hours and harvested at 72 hours.

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In the zymography assay, with the beginning of the specific experiment, control and latanoprost-treated cells were serum starved. Exposure to latanoprost occurred at 0, 24, and 48 hours. All cells were harvested at 72 hours.

**RNA Extraction and Gene-Specific RT-PCR**

RNA was isolated with extraction reagent (TRIzol; Sigma-Aldrich, Munich, Germany) according to the recommendations of the manufacturer and dissolved in diethyl pyrocarbonate (DEPC)-treated water. RNA was isolated with extraction reagent (TRIzol; Sigma-Aldrich, Munich, Germany) according to the recommendations of the manufacturer. We used the following PCR cycle parameters: hot-start polymerase activation for 15 minutes at 95°C, followed by 20 seconds at 95°C and up to 50 cycles at 95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 20 seconds. Detection of the fluorescence product was performed during the last 10% of the cycles. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis and subsequent agarose gel electrophoresis (data not shown). Genomic DNA contamination was excluded by control amplification reactions with nontranscribed RNA as templates. Only background fluorescence data were found. The quantification data were analyzed with the thermocycler system software (iCycler iQ; Bio-Rad Laboratories) as described.28

**Quantification of mRNA in Tenon Fibroblasts by Real-Time RT-PCR**

To detect the mRNAs and relative amount of different MMP and TIMP mRNAs in Tenon fibroblasts, real-time PCR was performed investigating MMP-1, -2, -3, -7, -9, and -14 and TIMP-1 and -2. In addition, the level of mRNA expression of MMP-3 and TIMP-2 in untreated cells and cells that were treated with latanoprost as described was quantified.

Real-time RT-PCR was performed using nucleic acid stain (SYBR Green I; Molecular Probes, Eugene, OR) on the thermocycler (iCycler; Bio-Rad Laboratories). Using the primer analysis software (Oligo 4.1; National Biosciences), we selected gene specific primers suitable for real-time RT-PCR, as shown in Table 1. The melting temperatures (T_m) of the primers were chosen between 58°C and 60°C wherever possible, and the expected fragment length lay between 79 and 178 bp. With these primers the mRNA expression of all genes together with GAPDH or B2MG as calibrators were analyzed simultaneously in a single experiment in triplicate reactions.

The analysis was repeated twice. Aliquots of the diluted cDNAs of all Tenon fibroblasts corresponding to 25 ng initially used total RNA, were mixed with 10× reaction buffer containing Tris-HCl and KCl, (NH4)2SO4, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.2 μM of each specific primer and 1 U of polymerase (HotStarTaq; Qiagen) in a volume of 50 μL.

The following PCR cycle parameters were used: hot-start polymerase activation for 15 minutes at 95°C and up to 50 cycles at 95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 20 seconds. Detection of the fluorescence product was performed during the last 10% of the cycles. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis and subsequent agarose gel electrophoresis (data not shown). Genomic DNA contamination was excluded by control amplification reactions with nontranscribed RNA as templates. Only background fluorescence data were found. The quantification data were analyzed with the thermocycler system software (iCycler iQ; Bio-Rad Laboratories) as described.28

The comparative Ct method was used for quantification of the target genes relative to GAPDH or B2MG.

**Immunocytochemistry**

Immunocytochemistry was performed as previously described.29 Briefly, untreated cells and latanoprost-treated cells as described were fixed for 10 minutes in acetone at −20°C. After incubation for 1 hour with mouse monoclonal anti-MMP-3 or mouse monoclonal anti-TIMP 2

### Table 1. Primers Used for Real-Time RT-Polymerase Chain Reaction to Detect Different MMPs and TIMPs in Tenon Fibroblasts

<table>
<thead>
<tr>
<th>Gene AC-Number</th>
<th>Primer Sequence (5′-3′), Position</th>
<th>Melting Temp (°C)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP1 (NM_002421)</td>
<td>TTC CTG GAA TTG GCC ACA AAG TT, pos. 1327</td>
<td>58.5</td>
<td>82</td>
</tr>
<tr>
<td>MMP2 (NM_004530)</td>
<td>GCA ATG CCA TTC CCG ATG ACCT, pos. 2118</td>
<td>60.7</td>
<td>151</td>
</tr>
<tr>
<td>MMP3 (NM_002422)</td>
<td>CGT GGA TGC CCG ATG TGA AT, pos. 1080</td>
<td>58.0</td>
<td>79</td>
</tr>
<tr>
<td>MMP7 (NM_002423)</td>
<td>CAG GCT GTA CTT CAG AGT TG, pos. 467</td>
<td>58.5</td>
<td>178</td>
</tr>
<tr>
<td>MMP9 (NM_004994)</td>
<td>AGG GGG AAG ATG CTG TCT A, pos. 1880</td>
<td>59.6</td>
<td>139</td>
</tr>
<tr>
<td>MMP14 (NM_004995)</td>
<td>TGG ATG CCC AAT GGA AAG ACC TA, pos. 1495</td>
<td>59.2</td>
<td>154</td>
</tr>
<tr>
<td>TIMP1 (NM_002534)</td>
<td>CCG GGG CTT CAC GAA CTA, pos. 470</td>
<td>59.8</td>
<td>111</td>
</tr>
<tr>
<td>TIMP2 (NM_003255)</td>
<td>GAG GAG GGC CCT GGT TAG ATA AAC, pos. 563</td>
<td>59.9</td>
<td>165</td>
</tr>
<tr>
<td>GAPDH (M35197)</td>
<td>GCT GGT GGT CCA TTC TCA TT, pos. 1064</td>
<td>59.3</td>
<td>154</td>
</tr>
</tbody>
</table>
(Oncogene, Boston, MA) diluted in PBS with 5 g/L BSA (mouse IgG or rabbit serum served as the control), two washes in PBS, we detected the immunoreactivity with Cy2-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) at 1:200 for 50 minutes. The cells were counterstained with Hoechst dye H33342 (Hoechst, Germany). For positive controls we used a mouse anti-β-actin antibody (Sigma-Aldrich).

**Western Blot Analysis**

Protein studies were performed as previously described in detail elsewhere. Twenty micrograms of protein per lane were separated on a 10% to 20% linear gradient SDS-PAGE gel (Bio-Rad) and electroblotted onto a nitrocellulose. Equal loading was ascertained by ponceau S staining. The primary monoclonal mouse antibodies were against MMP-3 (Ab-5; Oncogene) and TIMP-2 (Ab-2; Oncogene). Horseradish peroxidase-conjugated sheep anti-mouse antibody (1:3000; Amersham, Braunschweig, Germany) was used as the secondary antibody. Immunoreactivity was detected by chemiluminescence (Roche, Indianapolis, IN). For positive controls, the antigens MMP-3 (Biotrend, Cologne, Germany) with a weight of 57/59 kDa and TIMP-2 (Biotrend) with a weight of 21 to 22 kDa were used.

**Flow Cytometry**

Flow cytometry analysis was performed as previously described. Both untreated and latanoprost-treated cells were used. In brief, fibroblasts were rinsed in cold PBS, incubated for 5 minutes in trypsin at 37°C and harvested into complete medium containing 10% FCS. After centrifugation, the cells were percolated by resuspension in I test tubes in 75% ethanol for 10 minutes at 4°C. Cells were washed in cold PBS, incubated for 20 minutes in 10% rabbit serum in flow cytometry buffer (PBS/1% BSA/0.01% sodium azide) and incubating with 1 μg/ml mouse monoclonal antibody against MMP-3 (Ab-5) or TIMP-2 (Ab-2; Oncogene) at 4°C for 60 minutes. Isotype controls were incubated with 1 μg/ml nonspecific mouse IgG (Sigma-Aldrich). After washing in flow cytometry buffer the cells were incubated for 30 minutes at 4°C in FITC-conjugated rabbit anti-mouse IgG (Sigma-Aldrich). Cells (~10,000) were measured using flow cytometry (FacsCalibur Flow Cytometer; BD Biosciences, Heidelberg, Germany). The specific fluorescence index (SFI) was calculated as the ratio of the mean fluorescence obtained with the specific antibody and the isotype control.

**Zymography for MMP**

The casinoletic activity of untreated control cells and latanoprost-treated cells was shown by zymography, using 4% to 16% gels (Blue-Casein; Invitrogen). Conditioned medium was adjusted to the same quantity of cellular protein (75 μg). The medium was concentrated to 7 μL by a speed vacuum drier before use. The medium samples were treated with the same amount of sample buffer without boiling or reduction. SDS-PAGE was performed using a prestained prestain 4% to 16% polyacrylamide gel containing 0.1% casein at 125 V for 90 minutes. The gels were soaked in renaturation solution at room temperature and incubated in developing solution at 37°C overnight, as recommended by the manufacturer to allow proteinase digestion of its substrate. Proteolytic activities appeared as clear bands of lysis against a dark background of stained casein. The gels were stained with 0.5% Coomasie brilliant blue R-250 for 1 hour in 20% methanol and 10% acetic acid and destained in the same solution as described earlier, but without the dye, to visualize the molecular weight markers. The experiments were performed in triplicate.

**Animal Experiment**

Twelve female pigmented chinchilla outbred rabbits initially weighing 1.9 to 2.5 kg were studied. The experiment was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The project was approved by the local animal research review committee of the authors’ institution. The animals were held under a 12-hour day-night cycle. They were fed and had access to water ad libitum.

**Treatment**

The animals were randomly and equally divided into two groups of six. Only right eyes were treated. All left eyes were untreated and served as the control. Latanoprost eye drops (50 μg/ml; Xalatan; Pharmacia GmbH, Erlangen, Germany) were applied once per day. Treatment lasted 1 week (group 1) or 1 month (group 2).

**Histopathology**

After the treatment period, the rabbits were killed, and all right eyes were enucleated with sufficient amounts of peribulbar conjunctiva for analysis. Six left eyes were randomly selected as the control (three from each of the two different groups) and similarly enucleated and processed. For orientation, a silk suture was placed at the 12 o’clock position through the cornea. The globes were fixed in paraformaldehyde 4% buffered at pH 7.2 and embedded in paraffin for light microscopic examination and immunohistochemistry. Sections were prepared in the central vertical pupill–optic nerve head plane. The slides prepared included sections of both superior and inferior conjunctiva stained with hematoxylin-eosin.

**Immunohistochemistry**

For light microscopic immunohistochemistry, the labeled streptavidin-biotin method using a kit (LSAB Plus; Dako, Glostrup, Denmark) was applied on paraffin-embedded tissue sections according to the manufacturer’s instructions. Briefly, the sections were incubated with monoclonal mouse anti-rabbit MMP-3 and mouse anti-TIMP-2 antibodies (Chemicon, Temecula, CA) in a concentration of 5 μg/ml, with the biotinylated link antibody, and the horseradish peroxidase (HRP)-conjugated streptavidin, 30 minutes each. Proteolytic predigestion using proteinase K was performed for 6 minutes. 3-Amino 9-ethyl carbazole was used as a chromogenic substrate and Mayer’s hemalum as a counterstain. Negative control experiments included incubation of sections with irrelevant monoclonal antibodies and omission of the primary antibodies.

**RESULTS**

**RNA Extraction and Gene-Specific RT-PCR**

The mRNA expression of MMP-3 and TIMP-2 in untreated human Tenon fibroblasts and in latanoprost-treated cells of passage 3 was monitored by RT-PCR. On an ethidium bromide-stained agarose gel, the expected band length for the MMP-3 RT-PCR product (455 bp) and the TIMP-2 RT-PCR (612 bp) products was demonstrated (Fig. 1). MMP-3 as well as TIMP-2 mRNA was expressed in untreated human Tenon fibroblasts as well as in latanoprost-treated cells.
TABLE 2. Results of Real-Time RT-PCR Detecting the mRNA of MMP-3 and TIMP-2 in Untreated Fibroblasts and Cells Treated with Latanoprost for 1, 2, and 3 Days

<table>
<thead>
<tr>
<th>Cells</th>
<th>MMP-3</th>
<th>TIMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00 ± 0.39</td>
<td>1.00 ± 0.25</td>
</tr>
<tr>
<td>1 Day latanoprost</td>
<td>3.71 ± 1.52</td>
<td>4.75 ± 0.62</td>
</tr>
<tr>
<td>2 Days latanoprost</td>
<td>654 ± 78</td>
<td>4.20 ± 0.41</td>
</tr>
<tr>
<td>3 Days latanoprost</td>
<td>6280 ± 1704</td>
<td>2.29 ± 0.10</td>
</tr>
</tbody>
</table>

The values shown correspond to relative thresholds with the controls given a value of 1.00 (mean ± SD).

We varied the cDNA input into the RT-PCR from 62.5 ng (Fig. 1, lanes 1, 4, 7, 10) to 12.5 ng (lanes 2, 5, 8, 11), and 2.5 ng (lanes 3, 6, 9, 12) per reaction to get a semiquantitative impression of the MMP-3 and TIMP-2 mRNA expression level. Even on the lowest cDNA input, the band intensity was high (Fig. 1), and a clear difference in the band intensity was not discernible. We therefore used a quantitative real-time RT-PCR analysis for quantification of the MMP-3 and TIMP-2 mRNA level.

Real-Time RT-PCR

Including the same amount of cDNA (25 ng per reaction), real-time RT-PCR was used to quantify the mRNA levels of MMP-1, -2, -3, -7, -9, and -14 and TIMP-1, and -2 in untreated human Tenon fibroblasts. The log-linear portion of the fluorescence versus cycle plot was extended to determine a fractional cycle number at which a gene-specific threshold fluorescence was obtained (threshold cycle, Ct) for each analyzed gene and for GAPDH and B2MG as references. The threshold cycle for GAPDH as well as B2MG was equal in latanoprost-treated cells (21.40 ± 0.35 and 23.52 ± 0.92, respectively) and untreated cells (21.76 ± 0.36 and 24.24 ± 0.30, respectively). That shows that the expression of the reference genes is independent from latanoprost treatment.

In descending order, the threshold cycles of the analyzed MMPs and TIMPs were 22.1 (TIMP-1), 24.5 (MMP-2), 30.8 (TIMP-2), 31.8 (MMP-14), 38.1 (MMP-9), 42.3 (MMP-1), 42.8 (MMP-3), and 43.9 (MMP-7). Thus, TIMP-1 was the gene with the highest expression, comparable to GAPDH (CT of 21.4), and MMP-7 had the lowest expression. The other genes had an expression level between these values.

In a second step, the mRNA was in addition quantified in latanoprost-treated cells for MMP-3 and TIMP-2. These cells were exposed to latanoprost for 1, 2, or 3 days. In this experiment the mRNA expression was much more elevated as shown by the threshold cycle of 18.4 for untreated cells and 16.8, 16.2, and 16.0 for latanoprost-treated cells for 1, 2, and 3 days, respectively. Calibration of the MMP-3 and TIMP-2 expression with the GAPDH-expression by subtracting the gene-specific ΔCt of latanoprost-treated cells from the ΔCt of the untreated cells resulted in comparable mRNA expression (Table 2). There was a great increase in MMP-3 as well as TIMP-2 expression beginning with the 1-day treatment. The maximum MMP-3 expression was at day 3, whereas for TIMP-2 the maximum was at day 1 with a decrease on days 2 and 3.

These results from the PCR studies from untreated and latanoprost-treated fibroblasts demonstrate that the cells are able to produce various MMPs and TIMPs on the mRNA level. These MMPs and TIMPs are the important ones for tissue remodeling and wound healing.

Immunocytochemistry

With the technique described in the Methods section, positive staining with the antibodies against MMP-3 and TIMP-2 was present in most of the cultured cells of human Tenon fibroblasts used for the experiment (Figs. 2A, 2C). The labeling was mostly in the cytoplasm of the cells. These results demonstrate the presence of MMP-3 and TIMP-2 on the protein level. In cells treated with latanoprost, the staining in the cytoplasm was more intense (Figs. 2B, 2D). In the positive control samples, staining for β-actin was positive (results not shown).

Western Blot Analysis

With the help of this technique, specific bands for MMP-3 (Fig. 3) and TIMP-2 (Fig. 4) were detected. The positive control confirmed the band for MMP-3 to be at 57 kDa and the band for TIMP-2 to be at 57 kDa.
TIMP-2 to be at 21 kDa. These bands at the expected locations demonstrated the presence of the proteins in untreated Tenon fibroblasts. Latanoprost-treated cells showed a thicker band on the membrane, both for MMP-3 and TIMP-2, suggesting an increase of these proteins after treatment with the prostaglandin.

Flow Cytometry
The SFI increased from the untreated cells to the latanoprost-treated cells from 1.0 to 1.24 for MMP-3 and from 0.94 to 1.35 for TIMP-2 (Fig. 5). These results are representative of three experiments with similar results.

The results of the different techniques of immunocytochemistry, Western blot analysis, and flow cytometry demonstrate the presence of MMP-3 and TIMP-2 in Tenon fibroblasts on the protein level. Furthermore, the results indicate in all assays an increase of the respective proteins after latanoprost treatment.

Zymography
The caseinolytic activity of MMP-3 was demonstrated in the serum-free conditioned medium of samples from controls (Fig. 6, lanes 1, 2) and latanoprost-treated cells (Fig. 6, lanes 4, 5). Cells were treated with latanoprost for 3 days as indicated. A strong band was detected at 57 kDa, corresponding to MMP-3. The third band in latanoprost-treated cells may correspond to the 72-kDa band of MMP-2.

Animal Experiment
Histopathology. Examination of the sections stained with hematoxylin-eosin concentrated on the conjunctival histology

**FIGURE 5.** Flow cytometry of Tenon fibroblasts labeled with antibodies against MMP-3 and TIMP-2 in untreated and latanoprost-treated cells. Open trace: represents the control; solid area: specific staining.

**FIGURE 6.** Zymography for MMP-3. The caseinolytic activity of MMP-3 was demonstrated in the serum-free conditioned medium of samples from control cultures (lanes 1, 2) and latanoprost-treated cells (lanes 4, 5). Lane 3: molecular weight marker. The 72-kDa band in latanoprost-treated cells may correspond to MMP-2.

Immunohistochemistry. In control eyes, fibroblasts in the conjunctival stroma did not stain with the antibodies against MMP-3 and TIMP-2. In latanoprost-treated eyes, the basal epithelial cells showed an increased staining for MMP-3 and TIMP-2. In the stroma, a moderate number of fibrocytes were stained, whereas the extracellular matrix did not show any positive reaction (Fig. 7). This was true for eyes treated both for 1 and 4 weeks. The absence of positive staining in the control may be explained by the lower sensitivity of the immunohistochemical technique itself when compared with immunocytochemistry of tissue cultures and techniques such as Western blot or flow cytometry.

These results show that in the in vivo setting, the increase in MMP-3 and TIMP-2 after latanoprost treatment is also present.

**DISCUSSION**

The role of matrix metalloproteinases in glaucoma has not yet been fully elucidated. MMPs are a group of proteolytic enzymes active against all components of extracellular matrix. Despite their effects on Tenon fibroblasts, MMP and TIMP may play several roles in the context of glaucoma.

An increased expression of MMP-1 was found in retinal ganglion cells of glaucomatous eyes. In addition, MMP-1, -2, and MMP-3 were found all over the optic nerve heads and in the postlaminar region, especially in eyes with normal pressure glaucoma compared with control eyes. In monkeys, the

**FIGURE 7.** Immunohistochemistry for MMP-3 and TIMP-2. Immunohistochemistry of rabbit conjunctiva from control eyes (A, B) and eyes treated with latanoprost for 1 (C, D) and 4 (E, F) weeks once daily. (A, C, E) Staining for MMP-3. Compared with the control (A), there was positive staining for MMP-3 both at 1 (C) and 4 (E) weeks, in the basal epithelial cells (arrowheads) of the conjunctiva and in individual fibroblasts in the substantia propria (arrows). In addition, endothelial cells of capillaries and small vessels were positively stained. (B, D, F) Staining for TIMP-2. In the control, there was mild staining for TIMP-2 in the basal epithelial layer of the conjunctiva. Compared with the control (B), there was increased positive staining for TIMP-2, both at 1 (D) and at 4 (F) weeks in the basal epithelial cells (arrowheads) of the conjunctiva and in individual fibroblasts in the substantia propria (arrows). In addition, endothelial cells of capillaries and small vessels were positively stained.
amount of MMP-2 and -3 was increased in the anterior part of the ciliary muscle after application of latanoprost (a prostaglandin F2α analogue). It was speculated that this finding might correlate with an increased uveoscleral outflow pathway of aqueous humor. An increase of MMP-1 was found by others in the posterior uveal tract after exposure to latanoprost. These findings were expanded by others who revealed a strong reactivity for MMPs in the iris and the nonpigmented ciliary epithelium and in structures related to the uveoscleral outflow pathway, including the ciliary muscle and sclera. A mild reactivity was also noted in Schlemm’s canal. Several active forms of MMPs were found in aqueous humor of normal eyes, and an increase of TIMPs was found in the aqueous humor of eyes with primary open-angle glaucoma.

Returning to the issue of wound healing, we find the results of a study involving pterygia. In pterygia samples and cultured pterygium epithelial cells, an increase of both MMPs and TIMPs was found. This finding may indicate that the regulation of MMPs and TIMPs plays a role in the formation of excessive fibrous tissue that is produced by fibroblasts. In another study examining conjunctival scar tissue, MMPs and TIMPs were detected in the scar tissue, but not in the control tissue.

The goal of the current investigation was the thorough demonstration of the presence or absence of different MMPs and TIMPs in untreated human Tenon fibroblasts, in that the previous reports in the literature are not conclusive. In a second step, a possible increase in MMP and TIMP levels after exposure to a prostaglandin should be demonstrated.

To investigate the MMPs and TIMPs in Tenon fibroblasts at the RNA level, we performed a RT-PCR assay. With this technique, the presence of both enzymes was shown (Fig. 1). As mentioned earlier, more forms of MMPs and TIMPs exist, so that now we used the highly specific technique of the real-time RT-PCR. Two sets of primers were designed to detect the RNA of other MMPs and TIMPs that are related to collagen biosynthesis. mRNA of MMP-1, -2, -3, -7, -9, and -14 and TIMP-1 and -2 were detected. In addition, there was with the same assay that a treatment of the fibroblasts with latanoprost increased the amount of mRNA. This result suggests that the regulation of MMPs and TIMPs is controlled on the mRNA level.

For further investigations on the protein level, we performed immunocytochemistry, immunoblot analysis, and flow cytometry (Figs. 2, 3, 4, and 5). Positive staining with the antibodies against MMP-3 and TIMP-2 was seen in immunocytochemistry. With the immunoblot assay, specific bands on the gels were present for the same two proteins, and in addition, positively labeled cells were detected by flow cytometry. Each of these experiments alone, but also the sum of them showed that untreated Tenon fibroblasts contain both MMP-3 and TIMP-2. Furthermore, we also performed these assays with latanoprost-treated cells. We found an increase of MMP-3 and TIMP-2 in these cells. This indicates an increase in the amount of these proteins on the protein level after latanoprost treatment and is coincident with previously mentioned experiments elsewhere in the eye.

For MMP, the caseinolytic activity was shown with the zymography assay. This activity again was higher in the latanoprost-treated cells.

Finally, the results of various assays performed on human Tenon fibroblasts were confirmed in vivo with the rabbit experiment. After treatment with latanoprost at doses that are comparable to therapy for glaucoma, we detected an increase in immunostaining for both MMP-3 and TIMP-2 in the conjunctiva. These results were obtained after therapy for 1 week and 1 month. Therefore, it seems that, in addition to the results discussed earlier about the changes in the uveal tract, that the short-term application of latanoprost has an impact on the levels of MMPs and TIMPs in the conjunctiva. Recently, it was found that the increase of MMP levels in human ciliary muscle cells after application of latanoprost is caused by an increase of metalloproteinase gene transcription. With the results obtained in our study, it appears that this is similarly true of Tenon fibroblasts.

A clinical implication of these findings may be that it is favorable to use only topical antiglaucoma medications that have an effect similar to latanoprost on the level of MMPs and TIMPs. This may reduce tissue changes and therefore postoperative scarring after filtering surgical procedures.

A potential weakness of this study may be that these results are only from cell cultures and a well-controlled animal experiment. The impact of these effects in human conjunctiva is unclear. The effect may be even more difficult to determine, because most patients use more than one antiglaucoma medication before surgery, and it may be difficult to validate this effect in a clinical setting.

In summary, the results of our investigations indicate that Tenon fibroblasts may be capable of producing all forms of MMPs and TIMPs that are known to be related to extracellular matrix remodeling. In addition, these fibroblasts appear to be the target cells in an effort to influence the levels of these important proteins, and the application of latanoprost increases the secretion of MMPs and TIMPs. Controversial results of previous studies reporting the absence of MMPs and TIMPs in normal conjunctiva may be related to the technique, because, for example, immunostaining techniques may not be sensitive enough to detect small amounts of the proteins. An increase in both MMPs and TIMPs caused by antiglaucoma medication such as a specific prostaglandin may be beneficial and contribute to a reduced amount of postoperative scarring.

References


