Effect of Latanoprost on the Expression of Matrix Metalloproteinases and Tissue Inhibitor of Metalloproteinase 1 on the Ocular Surface

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Objective: To investigate the effect of topical latanoprost on the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase 1 (TIMP-1) on the ocular surface.

Methods: Tears were collected from 39 patients with glaucoma who used latanoprost, 0.005%, eyedrops (Xalatan) and 28 healthy volunteers. The MMP-9 concentration was measured. Conjunctival epithelial cells were collected from 10 eyes of 10 patients before and 1 to 3 months after starting to take topical latanoprost, 0.005%, and MMP-1, MMP-9, and TIMP-1 messenger RNA (mRNA) expression was analyzed. Both eyes of 48 mice were treated once a day with latanoprost, 0.005%, timolol gel, 0.5%, eyedrops, vehicle of Xalatan, or phosphate-buffered saline, and MMP-9 and TIMP-1 mRNA expression was analyzed.

Results: The median MMP-9 concentration in latanoprost-treated cases was 91.2 ng/mL (in controls, 19.7 ng/mL; P < .001). In latanoprost-treated cases, the relative ratio of MMP-9 to glyceraldehyde 3-phosphate dehydrogenase mRNA was significantly increased from 6.42 to 21.3 (P = .04, paired t test) and the relative amount of TIMP-1 was significantly decreased from 154 to 105 (P = .009). The relative amount of MMP-1 to GAPDH mRNA before and after latanoprost use was not significantly different (P = .16). In mice, MMP-9 expression was increased and TIMP-1 expression was decreased on the ocular surface at 8 weeks after latanoprost use.

Conclusion: The topical use of latanoprost increases MMP-1 and MMP-9 and decreases TIMP-1 on the ocular surface.

Clinical Relevance: The use of topical latanoprost might not be recommended in patients with keratoconus or after laser-assisted in situ keratomileusis.


LATANOPROST IS A PROSTAGLANDIN F2α analogue that is widely used to lower intraocular pressure in patients with glaucoma. The mechanisms underlying its effects to lower intraocular pressure include the upregulation of matrix metalloproteinases (MMPs) in the uveoscleral flow. Matrix metalloproteinases are a family of zinc-dependent endopeptidases that degrade the extracellular matrix. In many tissues, the ratio of MMPs and tissue inhibitors of metalloproteinases (TIMPs) determines the rate of extracellular matrix turnover. Latanoprost upregulates the expression of MMPs in cultured keratocytes and conjunctivae. Additionally, topical latanoprost upregulates MMP-9 in ciliary body smooth muscle, increases MMP-1 in choroid organ culture, and alters the expression of MMPs and TIMPs in the trabecular meshwork cells. These findings suggest that topical latanoprost alters the gene expression of MMPs and TIMPs on the ocular surface, which may affect the development and deterioration of some ocular surface diseases. In the present study, the effect of topical latanoprost on MMP and TIMP expression on the ocular surface was investigated in clinical cases and in an animal experiment.

METHODS

TEAR COLLECTION

Tears were collected from 39 eyes of 39 patients with glaucoma who used latanoprost eyedrops, 0.005% (Xalatan; Pfizer, New York, New York), for at least 1 month. The patients had primary open-angle glaucoma or normal-tension glaucoma and did not have ocular diseases other than refractive errors or slight physiologic cataract. They did not use other eyedrops and had no history of ocular surgery. Contact lens users were excluded. Control samples were obtained from 28 healthy age- and sex-
matched volunteers. To measure the MMP concentrations in a static condition, tears were collected with 3-µL micropipettes (disposable micropipettes, Drummond Scientific Company, Broomall, Pennsylvania) from a tear meniscus, with careful attention not to touch the ocular surface, which would cause reflex tear secretion. A new capillary tube was used for each patient. We collected 2 to 8 µL of tears. Tears were sealed in the microtube and frozen at −80°C as quickly as possible until analysis. This study was approved by the institutional review board of the University of Tokyo School of Medicine and adhered to the tenets of the Declaration of Helsinki.

**MEASUREMENT OF MMP-9 CONCENTRATION IN TEARS**

To measure the MMP-9 concentration in human tears, an enzyme-linked immunosorbent assay was performed using a commercially available kit (GF Healthcare UK Limited, Buckinghamshire, England). Samples of 2 to 8 µL, measured with a micropipette, were diluted to a final volume of 50 µL, and the assays were performed in accordance with the manufacturer’s instructions. The results are expressed as the concentration of the assay. All experimental procedures and statistical analyses were performed without knowledge of the patients’ clinical data.

**COLLECTION OF HUMAN CONJUNCTIVAL EPITHELIAL CELLS**

To examine the effect of latanoprost on MMP and TIMP-1 expression in the human conjunctival epithelium, another set of patients with glaucoma who were starting to take latanoprost, 0.005%, eyedrops were recruited. The patients had primary open-angle glaucoma or normal-tension glaucoma and did not have ocular diseases other than refractive errors or slight physiologic cataract. Ten eyes from 10 patients (1 man and 9 women; mean [SD] age, 57.2 [6.6] years) were examined. A brush cytology technique was used to collect the conjunctival epithelial cells. The surface of the palpebral conjunctiva was rubbed with a fine brush (Cytophush-5; Medscand, Monroe, Connecticut), and the brush was washed in a tube containing 1 mL of Isogen (Nippon Gene, Toyama, Japan). The tubes were stored at −80°C until RNA extraction. Samples were collected before and 1 or 3 months after starting latanoprost, 0.005%.

**RNA ISOLATION AND REVERSE TRANSCRIPTION TO COMPLEMENTARY DNA**

The RNA was isolated according to the manufacturer’s instructions. Total extracted RNA was reverse transcribed using a commercially available kit (Reverse Transcription System; Promega, Madison, Wisconsin) in accordance with the manufacturer’s instructions.

**QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION FOR HUMAN CONJUNCTIVAL SAMPLES**

The MMP-1, MMP-9, and TIMP-1 messenger RNA (mRNA) expression levels were analyzed with real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) using LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) with specific fluorescein hybridization probes, according to the manufacturer’s protocol. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were used to normalize the sample complementary DNA content. For MMP-9, the primers used were 5'-GAGGTCTTCCAGTGCCGCA-3' (forward) and 3'-CCTAGTCTCAGGGC-3' (reverse). The detection probes were labeled at the 5' end with LightCycler Red 640 (LC-Red) and modified by phosphorylation at the 3' end to block extension. The sequences of the probes were complementary to their antisense strand. The 3' fluorescein isothiocyanate (FITC)-labeled anchor probes were designed to hybridize to the antisense strand with a distance of 1 base 5' to the detection probes. The probes used were 5'-CAGCITGGCCACCTGTGCACTCCACTC-3' (FITC) and 5'-LCRed-GGAACCTGAGGCGCACAGTAA-GG-3'. The primers and probes for MMP-1, TIMP-1, and GAPDH were prepared with commercially available sets from Nihon Gene Research Laboratories (Sendai, Japan). The 20-µL PCR reaction mixture for MMP-9 contained 2.5 mM MgCl₂ with 0.3 µM of each primer, 0.1 µM of FITC probe, 0.2 µM of LC-Red probe, and 10% volume of LightCycler FastStart DNA master hybridization probes (Roche). The pipetting scheme for MMP-1, TIMP-1, and GAPDH was performed according to the manufacturer’s instructions. Polymerase chain reaction amplification began with a 10-minute denaturing step at 95°C, followed by 45 cycles of 10 seconds at 95°C, 15 seconds at 62°C (MMP-1, MMP-9, TIMP-1) or at 60°C (GAPDH); and 6 (MMP-9, TIMP-1), 8 (MMP-1), or 9 seconds (GAPDH) at 72°C. The fluorescein density of the LC-Red, reflecting the amount of the PCR product formed, was read at the end of each annealing step. Competitive RT-PCR was repeated 3 times for each sample.

**MOUSE EXPERIMENT**

Forty-eight ddY male mice (initially 10 weeks old) were obtained from Saitama Experimental Animals, Inc (Saitama, Japan). In this study, ddY mice were used because we had performed experiments examining the effect of latanoprost on mouse intraocular pressure and we knew that latanoprost was effective in reducing intraocular pressure in ddY mice. The experiment was performed in accordance with the ARVO statement for the use of animals in ophthalmic and vision research. The environment was maintained at 21°C with a 12-hour light and 12-hour dark cycle. All mice were fed ad libitum. The animals were randomly divided into 4 groups of 12. Both eyes of each group were treated once a day with 3 µL of latanoprost, 0.005%, eyedrops (group A), timolol-gel, 0.5%, eyedrops (Timoptol XE; Santen, Osaka, Japan) (group B), vehicle of Xalatan (group C), or phosphate-buffered saline (group D), respectively. The vehicle of Xalatan contained benzalkonium chloride, 0.02%, monosodium phosphate monohydrate, 0.5%, disodium hydrogen phosphate dihydrate, 0.6%, and sodium chloride, 0.4%. Treatment lasted for 4 weeks for half of the animals and 8 weeks for the other half (ie, 6 mice at 4 weeks and 6 mice at 8 weeks were used for each of 4 groups). After the treatment period, the mice were killed by cervical vertebrae dislocation under deep anesthesia following intraperitoneal injection of ketamine hydrochloride (100 mg/kg; Sankyo, Tokyo, Japan) and xylazine (10 mg/kg; Bayer, Munich, Germany). The eyes were enucleated bilaterally with sufficient conjunctiva for analysis, and the cornea and conjunctivae of 6 eyes of 6 mice in each group were dissected and homogenized in Isogen (Nippon gene). The RNA extraction and reverse transcription were performed as described above. Three pools of cDNA were created from each group, and the mRNA was evaluated with quantitative real-time RT-PCR. Protein was also extracted from 4 eyes of 4 mice in each group for MMP-9 Western blotting. Two eyes of 2 mice in each group were fixed in 10% formalin for immunohistochemistry. Both the administration of eyedrops and the following analyses were performed without knowledge of the eye being used.
QUANTITATIVE REAL-TIME RT-PCR FOR MOUSE SAMPLES

Quantitative analysis of mRNA in mouse samples was performed with quantitative real-time RT-PCR. The primers were 5’-CTCATGTACC GCCTGATAG-3’ (MMP-9, forward), 5’-GAGGATATGGTGGACACA-3’ (MMP-9, reverse), 5’-CGAGACCACCTTATACGCGC-3’ (TIMP-1, forward), and 5’-AATCTTCTT ATGGGTTCTG-3’ (TIMP-1, reverse). The probes were 5’-TGTTGCTGGCCTCTTAA GCTTGACCCAAG-3’-fluorescein (MMP-9, FITC), 5’-LCRed640-CCTCCAGCCCA CCACACAAGAACCC-3’-phosphorylation (MMP-9, LCRed), 5’-GAACGTGGG TTAGGGTACCCGATCTC-3’-fluorescein (TIMP-1, FITC), and 5’-LCRed460-CGG CATTTCCACAGCTTAACTC-3’-phosphorylation (TIMP-1, LCRed). The primers and probes for GAPDH were prepared with commercially available sets (Nikon Gene Research Laboratories).

The 20-µL PCR reaction mixture contained 3.0 mM (MMP-9) or 2.5 mM (TIMP-1) MgCl2 with 0.3 µM of each primer, 0.2 µM (MMP-9) or 0.1 µM (TIMP-1) FITC probe, 0.2 µM LC-Red probe, and 10% (vol/vol) LightCycler FastStart DNA master hybridization probes (Roche). The pipetting scheme for GAPDH followed the manufacturer’s instructions. The PCR amplification began with a 10-minute denaturing step at 95°C, followed by 45 cycles of 10 seconds at 95°C, 15 seconds at 62°C, or 9 seconds at 72°C (GAPDH). The melting curve of the PCR products was then confirmed. Competitive RT-PCR was repeated at least 3 times using 3 independent sample pools for each time point. Differences in the mean relative amount of mRNA were compared among the 4 groups.

IMMUNOHISTOCHEMISTRY

Formalin-fixed, paraffin-embedded sections were deparaffinized with xylene and rehydrated through a graded alcohol series. Antigen retrieval was performed with the heat-mediated method (boiling for 5 minutes with a microwave in sodium citrate buffer [pH, 6.0]). The samples were incubated with serum-free protein-blocking solution (Dako, Glostrup, Denmark) for 2 hours to block nonspecific sites. Sections were incubated at room temperature with anti–MMP-9 (1:500, ab38898; Abcam, Cambridge, Massachusetts) or anti–TIMP-1 (1:50, sc-5538; Santa Cruz Biotechnology, Santa Cruz, California) antibodies each for 2 hours. Sections were then extensively washed in phosphate-buffered saline with 0.3% Triton X-100 and then incubated with 488 µM conjugated antirabbit IgG (Invitrogen, Carlsbad, California) for 1 hour. After washing, sections were counterstained with propidium iodide (Vector Laboratories, Burlingame, California) for nuclear staining and mounted. Negative controls contained no primary antibody.

WESTERN BLOT ANALYSIS

Protein was extracted from the Isogen sample described in the real-time RT-PCR section following the manufacturer’s instructions, and protein concentrations were measured spectrophotometrically using a protein assay kit (Bio-Rad, Hercules, California). Equal amounts of protein (30 µg per lane) were dissolved in sample buffer, boiled for 5 minutes, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on Tris-HCl gradient gels, and blotted onto a polyvinyliden difluoride membrane (Bio-Rad) using the semidyed method. After incubation in blocking buffer, the polyvinylidene difluoride membrane was treated overnight with the diluted anti–MPP-9 antibody (1:1000, ab38898; Abcam, Cambridge, Massachusetts) and then with horseradish peroxidase–conjugated antirabbit immunoglobulin G (Bio-Rad) as the secondary antibody. A molecular weight standard (Precision Plus protein standard, No. 1610373; Bio-Rad) was used. The signal was detected by enhanced chemiluminescence (ECL Plus System; GE Healthcare) and was observed and analyzed with a computed image analyzer (LAS-4000; Fujifilm, Tokyo, Japan). The analysis was repeated 3 times using 3 independent samples.

STATISTICAL ANALYSIS

A Fisher exact test, paired t test, unpaired t test, or Mann-Whitney U test was used to compare the mean values and the distribution. Bonferroni correction for multiple comparisons was used when necessary. All analyses were performed using the Stat View statistical software package (SAS Inc, Cary, North Carolina). The level of significance was set to P < .05. All data are reported as the mean (standard deviation) unless otherwise specified.

RESULTS

MMP-9 PROTEIN IN HUMAN TEARS

The MMP-9 concentration in tears was measured in 39 eyes of 39 patients with glaucoma using topical latanoprost, 0.005% (17 men, 22 women; age, 49.0 [12.2] years), and 28 eyes of 28 healthy volunteers (7 men, 21 women; age, 53.5 [12.1] years). There were no significant differences in sex distribution (P = .13, Fisher exact test) or average age (P = .13, unpaired t test) between the 2 groups. Box and whisker plots with individual results are shown in Figure 1. Box and whisker plots were used because there were large interindividual differences in the glaucoma group. The median concentration of MMP-9 in the latanoprost-treated cases was 91.2 ng/mL (201 [243] ng/mL) and in controls was 19.7 ng/mL (22.7 [14.0] ng/mL; P < .001, Mann-Whitney U test).

MMPS AND TIMP-1 mRNA IN HUMAN CONJUNCTIVAL EPITHELIUM

Conjunctival epithelium was collected from 10 patients (1 man and 9 women). The mean (SD) age was 57.2 (6.5) years. Latanoprost use significantly increased the relative amount of MMP-9 to GAPDH mRNA from
6.42 (7.20) to 21.3 (21.5) (P = .04, paired t test) but significantly decreased the relative TIMP-1 levels from 154 (57.1) to 105 (30.7) (P = .009, paired t test). The relative amount of MMP-1 to GAPDH mRNA before (7.67 [4.60]) and after (6.86 [3.97]) latanoprost use was not significantly different (P = .16, paired t test).

mRNA AND PROTEIN OF MMP-9 AND TIMP-1 IN MICE

The relative amount of MMP-9 and TIMP-1 to GAPDH mRNA in the 4 groups of mice is shown in Figure 2. At 4 and 8 weeks, the relative amount of MMP-9 mRNA in group A was significantly greater than that of the other 3 groups. At 8 weeks, the relative amount of TIMP-1 mRNA in group A was significantly smaller than that of the groups C and D. * P < .05, † P < .01.

Figure 2. The relative amount of matrix metalloproteinase 9 (MMP-9) and tissue inhibitors of metalloproteinase 1 (TIMP-1) to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in 4 groups of mice. At 4 and 8 weeks, the relative amount of MMP-9 mRNA in group A was significantly greater than that of the other 3 groups. At 8 weeks, the relative amount of TIMP-1 mRNA in group A was significantly smaller than that of the groups C and D.

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Figure 3. Results of immunohistochemistry for matrix metalloproteinase 9 (MMP-9) in mice treated with latanoprost (A), timolol-gel (B), vehicle of Xalatan (C), phosphate-buffered saline (D), or negative control (E). A, In the latanoprost-treated group, immunostaining (green) for MMP-9 was intense in the epithelial cells, corneal stroma, and endothelial cells. B through D, In the 3 control groups, MMP-9 was detected in the nuclei of the epithelial cells in all layers (propidium iodide; objective magnification, ×400). Scale bar, 100 µm.

and Figure 4, respectively. Immunoreactivity to MMP-9 was detected in the nuclei of the epithelial cells of all layers in the 3 control groups, and staining for MMP-9 in the epithelial cells, corneal stroma, and endothelial cells was increased in the latanoprost-treated group. Immunoreactivity to TIMP-1 was detected in the cytoplasm of epithelial cells in the basal and suprabasal layers in the 3 control groups, and staining for TIMP-1 was decreased in the latanoprost-treated group. Although quantitative analysis could not be performed on the immunohistochemistry owing to its qualitative nature, these results appear to support the above-mentioned results in RT-PCR and add the data of locations where MMP-9 and TIMP-1 are produced.

Representative Western blot results for MMP-9 in the 3 groups of mice are shown in Figure 5A, and the results of quantitative analysis using a computer image analyzer are shown in Figure 5B. The increase in MMP-9 in the latanoprost-treated group was confirmed at the protein level.

Figure 4. Representative Western blot results for matrix metalloproteinase 9 (MMP-9) in the 3 groups of mice. The increase in MMP-9 in the latanoprost-treated group (B) was confirmed at the protein level compared with the vehicle (C) and negative control (D) groups.

The results of immunohistochemistry for MMP-9 and TIMP-1 in the 4 groups of mice are shown in Figure 3 and Figure 4, respectively. Immunoreactivity to MMP-9 was detected in the nuclei of the epithelial cells of all layers in the 3 control groups, and staining for MMP-9 in the epithelial cells, corneal stroma, and endothelial cells was increased in the latanoprost-treated group. Immunoreactivity to TIMP-1 was detected in the cytoplasm of epithelial cells in the basal and suprabasal layers in the 3 control groups, and staining for TIMP-1 was decreased in the latanoprost-treated group. Although quantitative analysis could not be performed on the immunohistochemistry owing to its qualitative nature, these results appear to support the above-mentioned results in RT-PCR and add the data of locations where MMP-9 and TIMP-1 are produced.

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The findings of the present study, based on analysis of human tear samples and epithelial cells, indicate that topical application of latanoprost increases MMP-9 in the tears and the conjunctivae and decreases TIMP-1 in the conjunctivae. Moreover, in mice, latanoprost increases MMP-9 and decreases TIMP-1 on the ocular surface. The al-
tered balance of MMPs and TIMPs induced by latanoprost appears to enhance ocular surface matrix degradation. These results are consistent with those of previous studies that describe altered expression of MMPs and TIMPs by latanoprost.1-8

Tears were collected with micropipettes from a tear meniscus, with careful attention not to touch the ocular surface, and 2 to 8 µL of tears was collected from each subject. Owing to the small volume of the samples and the detection limits for the measurable concentration of MMPs, only 1 kind of MMP could be measured. In previous studies, 15 to 20 µL of tear samples were collected. Because tear volume on the ocular surface is reportedly only 8 to 10 µL,10 the tear samples in the previous studies appear to have included reflex tear secretion, and thus the reported concentrations of molecules in the tears might differ from those obtained under physiologic conditions.

Commerically available latanoprost eyedrops (Xalatan) contain ingredients other than latanoprost such as preservatives (benzalkonium chloride) and pH-adjusting additives. These additional ingredients might affect MMP and TIMP expression. Thus, in mouse experiments, the vehicle of Xalatan (group C), A, TIMP-1 staining was decreased in the latanoprost-treated group. B through D, TIMP-1 was detected in the cytoplasm of epithelial cells in the basal and suprabasal layers in the 3 control groups (propidium iodide; objective magnification, ×400). Scale bar, 50 µm.

The results of this study suggest that the balance of MMPs and TIMPs altered by topical latanoprost leads to enhanced ocular surface matrix degradation. In accordance with this finding, a recent study reported that central corneal thickness was reduced by an average of 1.9% in patients who took topical latanoprost.11 Another clinical study, however, did not report major changes in corneal thickness after 1 year of use of topical latanoprost.12 There are a couple of possible reasons for this disagreement. First, because MMP-1, MMP-9, and TIMP-1 are only a small part of the total MMPs and TIMPs, the overall balance of MMPs and TIMPs on the ocular surface might not be altered by topical latanoprost. Comprehensive examination of the changes in MMPs and TIMPs induced by latanoprost on the ocular surface are needed to confirm this point. Second, corneal changes in clinical cases that used topical latanoprost might not have been followed up for a long enough period. Further observation with an extended long-term follow-up of clinical cases may be necessary.

Expression of MMP and TIMP has been investigated in various ocular surface diseases such as keratoconus13-16 and corneal ulcer.17,18 Because corneal thinning is a major finding in keratoconus, the relation between MMPs/TIMPs and keratoconus has been investigated. Most studies report that upregulation of MMPs and active forms of MMPs are not observed in corneas with kera-
On the other hand, Lema et al\textsuperscript{16} reported a higher concentration of MMP-9 in the tears of patients with keratoconus in relation to its severity. Taken together, these findings indicate that MMPs may not have a causative role in the development of keratoconus, but may be related to its deterioration. In agreement with this notion, we experienced a case of a 47-year-old in which keratoconus progressed rapidly while using topical latanoprost.\textsuperscript{19} Although this progression of keratoconus might be a natural course, it may be that latanoprost increased this progression through the upregulation of MMPs on the cornea. Based on this case and the findings of the present study, the use of topical latanoprost in cases with keratoconus might not be recommended. This may have a more striking effect in patients who have laser in situ keratomileusis in which corneal thickness is reduced by laser ablation and keratectasia sometimes occurs, simply because of the enormous number of patients who have the procedure. Although we did not show direct evidence that the altered balance of MMPs and TIMPs induced by latanoprost enhances ocular surface matrix degradation, topical latanoprost should be used cautiously in cases after laser in situ keratomileusis. The effect of latanoprost on corneas after laser in situ keratomileusis needs to be examined in a further clinical study.

The present study has some limitations. First, the number of studied enzymes was limited. Enzymes MMP-1, MMP-9, and TIMP-1 comprise only a small number of the inflammatory and remodeling proteins on the ocular surface. In this study, the volume of tear samples from human volunteers was too small to allow for the comprehensive examination of changes in MMP and TIMP levels induced by latanoprost that are needed to understand the overall effect of latanoprost on the ocular surface. Second, the effect of such a fluctuation in MMP levels as observed in this study may vary in clinical settings. The examination of MMP and TIMP levels in patients with ocular surface diseases in a large-scale study might provide more conclusive answers.

In conclusion, the topical use of latanoprost increases MMP-1 and MMP-9 and decreases TIMP-1 on the ocular surface. The altered balance of MMPs and TIMPs induced by latanoprost might enhance ocular surface matrix degradation.

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