

Effect of Bimatoprost, Latanoprost, and Unoprostone on Matrix Metalloproteinases and Their Inhibitors in Human Ciliary Body Smooth Muscle Cells

Yen Hoong Ooi, Dong-Jin Oh, and Douglas J. Rhee

PURPOSE. Matrix metalloproteinase (MMP)-mediated turnover of extracellular matrix (ECM) affects outflow resistance in the uveoscleral pathway. The balance of MMPs and tissue inhibitors of metalloproteinases (TIMPs) governs the rate of ECM turnover in many tissues. The hypothesis was that a differential effect on MMPs and TIMPs in ciliary body smooth muscle (CBSM) cells would relate to the relative intraocular pressure-lowering effectiveness of the prostaglandin analogues (PGAs) bimatoprost, latanoprost, and unoprostone.

METHODS. Human CBSM cells isolated from donor corneoscleral rims were incubated for 24 hours with control (0.015% ethanol in DMEM) or the free acid forms of bimatoprost (0.01 or 0.1 $\mu\text{g}/\text{mL}$), latanoprost (0.03 or 0.3 $\mu\text{g}/\text{mL}$), or unoprostone (0.145 or 1.45 $\mu\text{g}/\text{mL}$). Western blot analysis determined the relative protein concentrations of MMP-1, -2, -3, -9, and -24 as well as TIMP-1 through -4. Zymography measured the relative activity levels of MMP-1, -2, -3, and -9.

RESULTS. All PGAs increased MMP-1, -3, and -9. Bimatoprost and latanoprost did not change MMP-2. Unoprostone decreased MMP-2 (21% \pm 3%). On zymography, MMP-1 and -2 did not change. Bimatoprost and latanoprost increased MMP-9 activity by 75% \pm 27% and 75% \pm 24%, respectively. MMP-3 activity was not detected on zymography. All PGAs increased TIMP-3, but only unoprostone increased TIMPs1 and -4 by 100% \pm 20% and 61% \pm 11%, respectively. TIMP-2 was unchanged by bimatoprost and latanoprost, but decreased by unoprostone (35% \pm 8%).

CONCLUSIONS. Decreased MMP-2 with concurrent increases of TIMP-1 and -4 by unoprostone may explain the lower clinical efficacy of unoprostone. The MMP/TIMP balance relates to the observed intraocular pressure-lowering effectiveness in clinical studies with PGAs. (*Invest Ophthalmol Vis Sci.* 2009;50:5259-5265) DOI:10.1167/iovs.08-3356

Elevated intraocular pressure (IOP) is a major risk factor in the pathogenesis of glaucoma. Aqueous humor drains through the conventional pathway (trabecular meshwork, Schlemm's canal, collecting channels, and episcleral venous system) and alternative pathway (ciliary body face, suprachor-

oidal space with diffusion through sclera, and larger molecules via vortex venous system).

Within the ciliary body stroma portion of the uveoscleral tract, outflow resistance can be modulated by both ciliary smooth muscle cell tone¹⁻⁴ and enhanced turnover of extracellular matrix (ECM) by matrix metalloproteinases (MMPs).⁵⁻¹² We previously determined that the mRNAs of MMP-1, -2, -3, -11, -12, -14, -15, -16, -17, -19, and -24 as well as tissue inhibitors of metalloproteinase (TIMP)-1 through -4 are present in ciliary body and ciliary body smooth muscle (CBSM) cells.¹² In other human tissues, the ratio of MMPs to TIMPs correlates to the rate of ECM turnover.¹³⁻²¹

PGAs, such as bimatoprost, latanoprost, and unoprostone, treat glaucoma by lowering intraocular pressure. They affect aqueous drainage, but vary in the degree to which uveoscleral or conventional pathways are affected.²²⁻²⁵ All three are prostaglandin F_{2 α} (FP) receptor agonists.²⁶⁻³⁰ Numerous studies have found a greater IOP-lowering ability of latanoprost than that of unoprostone.³¹⁻³⁷ Some studies have found bimatoprost to have a greater IOP-lowering effect than latanoprost,^{38,39} but others have found them to be equivalent.⁴⁰

We hypothesized that the different PGAs would have differing effects on the MMP/TIMP balance in CBSM cells that relate to their relative effectiveness in lowering IOP.

METHODS

Tissue and Explant Culture

CBSM cells were cultured according to previously published protocols.^{12,41} Briefly, all ciliary body (CB) tissue was dissected from human donor corneoscleral buttons from the Massachusetts Eye and Ear Infirmary within 6 hours of corneal transplant surgery; these corneoscleral buttons contain significant CBSM cells.⁴² Information provided by the eye bank indicated the donors' age and nonglaucomatous status. CBSM cell cultures were generated from CB isolated from 13 separate individuals, ages 23 to 65 years. The cells' identity was confirmed by labeling with anti-desimin and anti-smooth muscle actin antibodies, as originally described by Weinreb et al.,⁴¹ and confirmed that >99% of cells in culture were CBSM cells. Furthermore, the cells had the usual morphologic appearance with individual cells having a spindle shape. Once confluent, CBSM cells grow in bundles mimicking a muscular pattern. The cultures were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) containing 20% fetal bovine serum, 1% L-glutamine (2 mM), and gentamicin (0.1 mg/mL) at 37°C in a 10% CO₂ atmosphere. All the cells used were from passage-4 and -5 cultures.

Bimatoprost, Latanoprost, and Unoprostone Incubation

The free acid forms of bimatoprost, latanoprost, and unoprostone (Cayman Chemical, Ann Arbor, MI) were prepared in ethanol and diluted to experimental concentrations with serum-free medium. CBSM cells (1 \times 10⁶) were plated into T-75 flasks and allowed to grow to confluence with medium changes every 3 to 5 days. Once confluent, the cultures were trypsinized into seven 60-mm dishes and allowed to

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Supported by Allergan, American Glaucoma Society Clinician-Scientist Fellowship, Massachusetts Lions Eye Research Foundation, Pfizer Ophthalmics, and EY 14104 (MEEI Vision-Core Grant).

Submitted for publication December 25, 2008; revised March 10, 2009; accepted September 1, 2009.

Disclosure: **Y.H. Ooi**, None; **D.-J. Oh**, None; **D.J. Rhee**, Allergan (F, R), Pfizer Ophthalmic (F, R)

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TABLE 1. Primary Antibodies and Their Dilutions Used for Immunoblot Analysis

Antibodies	Manufacturer*	Dilution	Identified Band Size(s)
MMP-1 (M)	R&D Systems	1:1,000	52 and 57 kDa
MMP-2 (M)	R&D Systems	1:1,000	72 kDa
MMP-3 (P)	Chemicon	1:1,000	57 and 59 kDa
MMP-9 (P)	Chemicon	1:1,000	92 kDa
MMP-24 (P)	Chemicon	1:1,000	64 kDa
TIMP-1 (P)	Chemicon	1:20,000	28 kDa
TIMP-2 (P)	Chemicon	1:2,000	21 kDa
TIMP-3 (M)	Calbiochem	1:200	27 kDa
TIMP-4 (P)	R&D Systems	1:7,000	29 kDa
GAPDH (P)	R&D Systems	1:20,000–40,000	36 kDa

M, monoclonal mouse anti-human antibody; P, polyclonal rabbit anti-human antibody.

* R&D Systems, Minneapolis, MN; Chemicon, Temecula, CA; Calbiochem, San Diego, CA.

grow to confluence. The cells were then incubated with serum-free medium for 24 hours and exposed to vehicle control (0.015% ethanol), 0.01 or 0.1 $\mu\text{g}/\text{mL}$ free acid bimatoprost, 0.03 or 0.3 $\mu\text{g}/\text{mL}$ free acid latanoprost, or 0.145 or 1.45 $\mu\text{g}/\text{mL}$ unoprostone, for 24 hours. The concentrations of these prostaglandin analogues (PGAs) were chosen based on the peak aqueous concentrations of these drugs after topical administration in humans (0.009 $\mu\text{g}/\text{mL}$, 0.028 $\mu\text{g}/\text{mL}$, and 0.145 $\mu\text{g}/\text{mL}$ for bimatoprost, latanoprost, and unoprostone, respectively).^{43–45} In our previous work with latanoprost, we have found that 0.3 $\mu\text{g}/\text{mL}$ (or 10 times the peak aqueous concentration) can help confirm the trend seen at 0.03 $\mu\text{g}/\text{mL}$, but a higher concentration of 30 $\mu\text{g}/\text{mL}$ did not provide further useful information.¹²

Cell Lysate and Conditioned Medium Preparation

Cultures of CBSM cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% SDS) with protease inhibitors (10 $\mu\text{L}/\text{mL}$ aprotinin, 0.1 mg/mL phenylmethylsulfonyl fluoride (PMSF), 1 mM EGTA, 1 $\mu\text{g}/\text{mL}$ leupeptin, and 1 mM sodium orthovanadate to a final volume of 1 mL with RIPA buffer). A 21-gauge needle was used to shear the cells thoroughly and an additional 10 of 10 mg/mL PMSF was added before incubation on ice for 30 to 60 minutes. After incubation, the cell lysate was centrifuged at 14,000g for 10 minutes at 4°C. The supernatant was collected as whole-cell lysate.

The conditioned culture media were collected every 24 hours after incubation with vehicle control and PGAs and concentrated 30-fold (Amicon Ultra-4, 10K; Millipore, Milford, MA). The protein concentrations were determined according to a protein assay protocol (BioMate; Thermo Spectronic, Rochester, NY). Ten microliters of each sample and RIPA with inhibitors as control were measured and compared with a previously established bovine serum albumin (BSA) curve at a 655-nm wavelength. The sample was aliquoted and stored frozen at -80°C until it was analyzed.

Western Blot Analysis

Equal amounts of protein from whole-cell lysates or concentrated conditioned media were electrophoresed into 11% SDS-PAGE gels (XCell SureLock Mini-Cell; Invitrogen). The separated proteins were blotted onto a nitrocellulose membrane with 0.45 μm pore size (Invitrogen). The membrane was blocked in blocking buffer (Rockland, Gilbertsville, PA) and 1× Tris-buffered saline (TBS) followed by primary antibody (Table 1) incubation overnight at 4°C. The following day, they were washed with TBS/T, the membranes were incubated with IRDye 800-conjugated affinity purified anti-mouse or anti-rabbit IgG (Rockland, Gilbertsville, PA) diluted 1:10,000 for 1 hour. The membranes were washed with 1× TBS with 0.1% Tween 20 (TBS/T) and scanned (Odyssey Infrared Imaging System; Li-Cor; Lincoln, NE).

The bands were quantified by using densitometric software (Odyssey; Li-Cor).

Zymographic Analysis

Zymography analyzes the ability of MMP-2 and -9 to degrade gelatin and MMP-1 and -3 to degrade casein as a measure of enzymatic activity. Gelatin (0.1%) or β -casein (0.1%) was mixed into liquid acrylamide when casting polyacrylamide gels. Concentrated conditioned medium, mixed with 2× Tris-glycine-SDS zymography sample buffer at 1:10 ratio, was loaded into 10% SDS-PAGE gels. The samples were electrophoresed at 130 V in tank buffer. The gels were washed at room temperature with 2.5% Triton X-100 (renaturing buffer), then transferred to development buffer overnight at 37°C. The resultant gels were stained with 0.1% Coomassie brilliant blue G-250 (Bio-Rad, Hercules, CA) solution for at least 3 hours, then destained with fixing/destaining solution until clear bands were visible and contrasted well with the blue background. The gels were scanned and analyzed for relative densities (Odyssey Infrared Imaging System; Li-Cor). All the MMPs were identified based on their molecular weights resolved by zymography and were confirmed by purified MMP-1, -2, -3, and -9 (Chemicon, Temecula, CA) as positive controls.

Determination of Change

We determined the variability of our Western blot by repeating the immunoblot analysis nine separate times, which revealed an average difference of $10.05\% \pm 9.45\%$. Thus, any change greater than 10% was defined as an elevation or decrease for the individual sample. According to published criteria, the effect of the PGA was determined by the trend of the majority (>60%) of the samples.^{7,12,46,47}

RESULTS

Effect on MMP and TIMP Levels

MMPs -1, -2, -3, and -9 are secreted proteins and were identified in conditioned media; we were not able to detect them from cell scrapings. Conversely, MMP-24 (MT5-MMP) was membrane bound and found in cell scrapings, but was not detectable in conditioned media. Results are reported for the three PGAs in alphabetical order, bimatoprost (BIM), latanoprost (LAT), and unoprostone (UNO), and grouped by MMP subfamilies of collagenases, gelatinases, stromelysins, and membrane bound. Pharmacologic doses for BIM, LAT, and UNO were 0.01, 0.03, and 0.145 $\mu\text{g}/\text{mL}$, respectively, while suprapharmacologic doses were 10 times the peak pharmacologic concentrations.

Pro-MMPs -1, -3, and -9 were all increased by BIM, LAT, and UNO. Pro-MMP-1 (collagenase-1) increased an average of $24\% \pm 6\%$ in four of five donors, $20\% \pm 3\%$ in three of five donors, and $23\% \pm 4\%$ in three of five in donors in response to BIM, LAT, and UNO (Table 2, Fig. 1A), respectively. At supratherapeutic doses of BIM, collagenase-1 increased an average of $27\% \pm 15\%$ in three of five donors and did not change in the other two donors. Collagenase-1 did not change in three of five donors in response to supratherapeutic doses of LAT and UNO.

BIM and LAT did not change pro-MMP-2 (gelatinase A) in four of five donors (Table 2, Fig. 1B). UNO decreased pro-MMP-2 an average of $21\% \pm 3\%$ at pharmacologic doses in three of five donors. Suprapharmacologic concentrations did not alter pro-MMP-2 in response to any of the PGAs in all five donors.

Pro-MMP-3 (stromelysin-1) increased $63\% \pm 10\%$, $30\% \pm 5\%$, and $65\% \pm 18\%$ in three of five donors in response to BIM, LAT, and UNO (Table 2, Fig. 1C), respectively. At suprapharmacologic doses of BIM and UNO, stromelysin-1 did not alter the protein level in three of five donors. Stromelysin-1 increased an average of $31\% \pm 6\%$ in three of five donors in

TABLE 2. Summary of the Effect of PGAs on the Expression of MMPs and TIMPs from the Conditioned Media of Human CBSM Cells

MMPs/TIMPs*	Bimatoprost	Latanoprost	Unoprostone
MMPs			
Pro-MMP-1	↑ 4/1/0 (24 ± 6) (20 ± 7)	↑ 3/2/0 (20 ± 4) (16 ± 4)	↑ 3/2/0 (23 ± 4) (13 ± 7)
Pro-MMP-2	↔ 0/4/1 (-2 ± 4)	↔ 0/4/1 (-3 ± 5)	↓ 0/2/3 (-21 ± 3) (-12 ± 6)
Pro-MMP-3	↑ 3/2/0 (63 ± 10) (35 ± 18)	↑ 3/2/0 (30 ± 5) (16 ± 9)	↑ 3/2/0 (65 ± 18) (35 ± 22)
Pro-MMP-9	↑ 3/1/1 (75 ± 27) (43 ± 28)	↑ 3/2/0 (76 ± 24) (45 ± 26)	↑ 3/0/2 (107 ± 53) (51 ± 50)
MMP-24 (CL)	↔ 0/3/2 (-9 ± 5)	↔ 0/5/0 (-5 ± 3)	↔ 0/3/2 (-9 ± 3)
Active MMPs			
Inter MMP-1	↔ 1/4/0 (7 ± 2)	↔ 1/4/0 (6 ± 2)	↔ 1/4/0 (5 ± 2)
Active MMP-2	↔ 0/5/0 (5 ± 1)	↔ 0/5/0 (3 ± 1)	↔ 0/5/0 (-1 ± 1)
Active MMP-9	↑ 3/2/0 (18 ± 3) (8 ± 5)	↑ 4/1/0 (19 ± 4) (13 ± 6)	Ind 2/1/2 (0 ± 7)
TIMPs			
TIMP-1	Ind 1/2/2 (-8 ± 21)	↓ 1/1/3 (-35 ± 16) (1 ± 31)	↑ 3/0/2 (100 ± 20) (37 ± 41)
TIMP-2	↔ 0/3/2 (3 ± 11)	↔ 0/3/2 (-11 ± 8)	↓ 0/2/3 (-35 ± 8) (-24 ± 9)
TIMP-3	↑ 3/0/2 (57 ± 23) (21 ± 28)	↑ 4/1/0 (69 ± 15) (55 ± 19)	↑ 3/1/1 (57 ± 9) (24 ± 21)
TIMP-4	ind 1/2/2 (2 ± 14)	↔ 1/3/1 (1 ± 12)	↑ 4/0/1 (61 ± 11) (42 ± 21)
TIMP-4 (CL)	↓ 2/0/3 (-24 ± 4) (9 ± 21)	↑ 3/2/0 (19 ± 7) (11 ± 6)	ind 2/1/2 (38 ± 36)

The cells were incubated with bimatoprost, latanoprost, and unoprostone at the pharmacologic concentrations 0.01, 0.03, and 0.145 $\mu\text{g}/\text{mL}$, respectively, for 24 hours. For MMP-24, the protein was isolated from cell scrapings.

* The data are represented as determination of effect (\uparrow , \downarrow , \leftrightarrow , ind); number of samples with densitometry greater than 10% of the respective control/number of samples within 10% of the control (i.e. defined as unchanged)/and number of samples with densitometry more than 10% below controls; (average % increase or % decrease \pm SE). The average percentage of changes for all samples is also presented on the next line; (average % increase or % decrease \pm SE). Ind, indeterminate; CL, protein harvested from cell lysates.

response to suprapharmacologic doses of LAT and did not alter in the other two donors.

Pro-MMP-9 (gelatinase B) increased $75\% \pm 27\%$, $76\% \pm 24\%$, and $107\% \pm 53\%$ in three of five donors in response to BIM, LAT, and UNO, respectively (Table 2, Fig. 1D). At suprapharmacologic doses, BIM increased gelatinase B by $51\% \pm 15\%$ in three of five donors but did not alter the protein level in two donors; LAT increased pro-MMP-9 by $47\% \pm 25\%$ in four of five

donors, whereas UNO decreased pro-MMP-9 by $33\% \pm 5\%$ in three of five donors.

Pharmacologic doses of BIM, LAT, and UNO, did not alter MMP-24 (MT5-MMP) in three of five, five of five, and three of five donors, respectively (Table 2, Fig. 1E). At suprapharmacologic doses, LAT decreased MMP-24 by $17\% \pm 1\%$ in three of five donors; BIM and UNO did not change MMP-24 levels in four of five and three of five donors, respectively.

CBSM

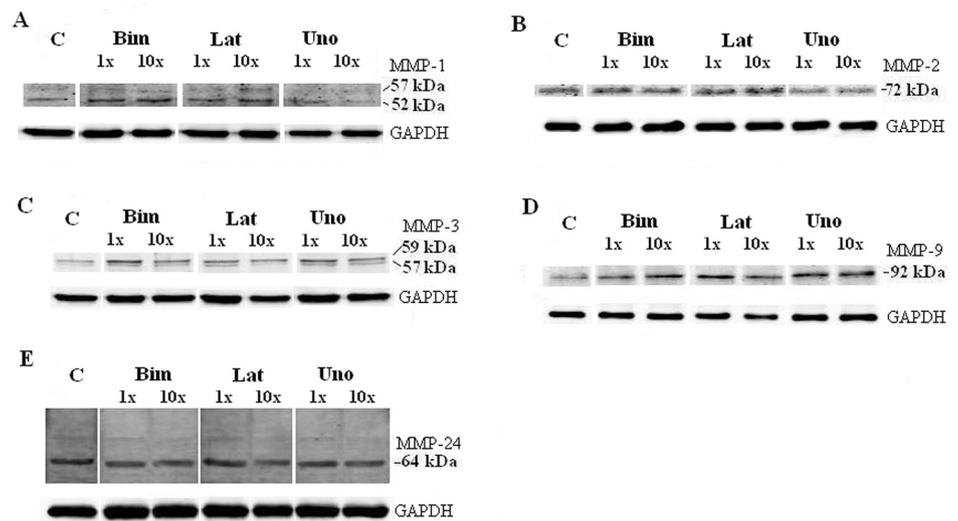


FIGURE 1. Representative Western blots for MMP-1 (A), -2 (B), -3 (C), -9 (D), and -24 (E) from the conditioned media of human CBSM cells incubated with bimatoprost, latanoprost, and unoprostone for 24 hours. C, control; 1 \times and 10 \times , samples incubated with the peak aqueous concentrations and 10 times the peak aqueous concentration of these drugs, respectively, for 24 hours.

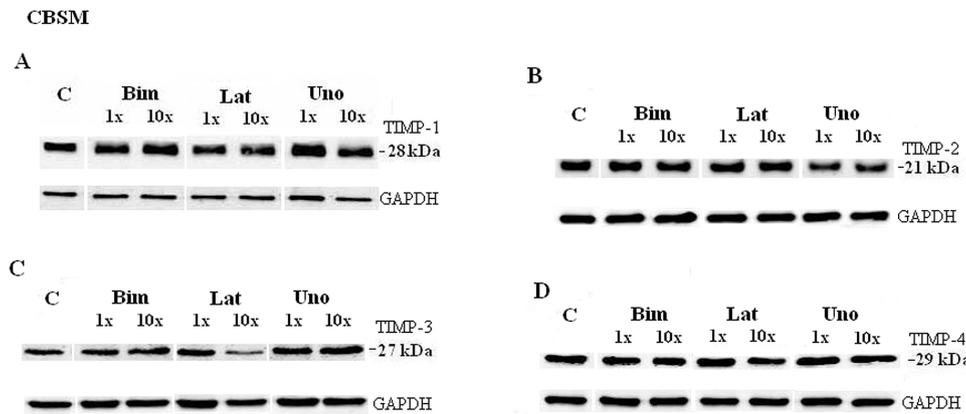


FIGURE 2. Representative Western blots of TIMP-1 (A), -2 (B), -3 (C), and -4 (D) from conditioned media in human CBSM cells incubated with bimatoprost, latanoprost, and unoprostone for 24 hours. C, control; 1× and 10× represent samples incubated with the peak aqueous concentrations and 10 times the peak aqueous concentration of these drugs, respectively, for 24 hours.

All TIMPs were found in both conditioned media and cell lysates. However in cell lysates, the basal levels of TIMP-1, -2, and -3 are consistently close to the lowest limit of detection; PGAs did not significantly change the levels of the aforementioned TIMPs in the cell lysates. In cell lysates, TIMP-4 decreased an average of $24\% \pm 4\%$ in three of five donors in response to BIM. LAT increased TIMP-4 an average of $19\% \pm 7\%$ in three of five donors. UNO increased TIMP-4 by $125\% \pm 4\%$ in two donors, decreased it $29\% \pm 5\%$ in another two donors, and had no effect on it in the fifth donor; given no clear majority, the effect of UNO on TIMP-4 in cell lysates was deemed indeterminate. At suprapharmacologic doses, BIM increased TIMP-4 an average of $69\% \pm 48\%$ in three of five donors but decreased it $18\% \pm 2\%$ in response to LAT in three of five donors. Suprapharmacologic doses of UNO increased TIMP-4 by $70\% \pm 37\%$ of in three donors but had no effect on it in the other two.

In conditioned media with pharmacologic concentrations, BIM decreased TIMP-1 an average of $55\% \pm 6\%$ in two of five donors and had no effect on it in the other three. Given no clear majority, the effect of BIM was recorded as indeterminate. LAT decreased TIMP-1 (Table 2, Fig. 2A) an average of $35\% \pm 16\%$ in three of five donors. UNO increased TIMP-1 an average of $100\% \pm 20\%$ in three of five donors. TIMP-2 (Table 2, Fig. 2B) was not altered by BIM or LAT in three of five donors, but decreased an average of $35\% \pm 8\%$ in three of five donors in response to UNO. TIMP-3 (Table 2, Fig. 2C) increased $57\% \pm 23\%$ in three of five donors, $70\% \pm 15\%$ in four of five donors, and $57\% \pm 9\%$ in three of five donors in response to BIM, LAT, and UNO, respectively. TIMP-4 was not changed in two of five donors, decreased an average of $23\% \pm 10\%$ in two donors, and increased in the remaining donor in response to BIM, which was an indeterminate result, given the lack of a majority response. LAT did not alter it in three of five donors (Table 2, Fig. 2D), but it increased $61\% \pm 11\%$ in four of five donors in response to UNO.

At suprapharmacologic doses in conditioned media, BIM increased TIMP-1 in three of five donors by $70\% \pm 31\%$. LAT did not change TIMP-1 in three of five donors. UNO increased TIMP-1 an average of $82\% \pm 40\%$ in two donors, decreased it by $61\% \pm 2\%$ in two donors, and had no effect in the remaining one. With no clear majority, the effect was defined as indeterminate. BIM did not change TIMP-2 in three of five donors and decreased it an average of $28\% \pm 4\%$ in the other two. LAT increased TIMP-2 an average of $39\% \pm 9\%$, whereas UNO decreased it an average of $30\% \pm 2\%$ in three of five donors. BIM and UNO increased TIMP-3 by $35\% \pm 9\%$ and $325\% \pm 158\%$ in three of five donors, respectively. LAT did not change TIMP-3 in two of five donors, decreased it an average of $37\% \pm 5\%$ in two others, and had no effect on the level in the fifth one. Thus, the effect of LAT on TIMP-3 was indeterminate. TIMP-4

decreased an average of $40\% \pm 7\%$ in all five donors in response to BIM. LAT decreased an average of $30\% \pm 4\%$ in three of five donors; UNO increased TIMP-4 an average of $28\% \pm 8\%$ in three of five donors.

In summary, all three PGAs had similar effects on MMPs, although UNO decreased MMP-2 compared to BIM and LAT. However, UNO upregulated more of the TIMPs than either LAT or BIM.

Effect on MMP Activity

Enzymatic activity of intermediate MMP-1 and -2 remained within 10% of control at pharmacologic and suprapharmacologic doses of all studied PGAs in four of five donors (Table 2, Figs. 3A, 3B). At pharmacologic doses, BIM increased MMP-9 activity by $18\% \pm 3\%$ in three of five donors; LAT increased it an average of $19\% \pm 4\%$ in four of five donors (Table 2, Fig. 3C). UNO did not change MMP-9 activity in two of five donors, decreased it by $16\% \pm 1\%$ in two others, and had no effect in the fifth donor; thus, the result was indeterminate. At suprapharmacologic doses, MMP-9 activity increased by $18\% \pm 3\%$ and $18\% \pm 4\%$ in four of five donors in response to BIM and LAT, respectively, but did not change in four of five donors in response to UNO.

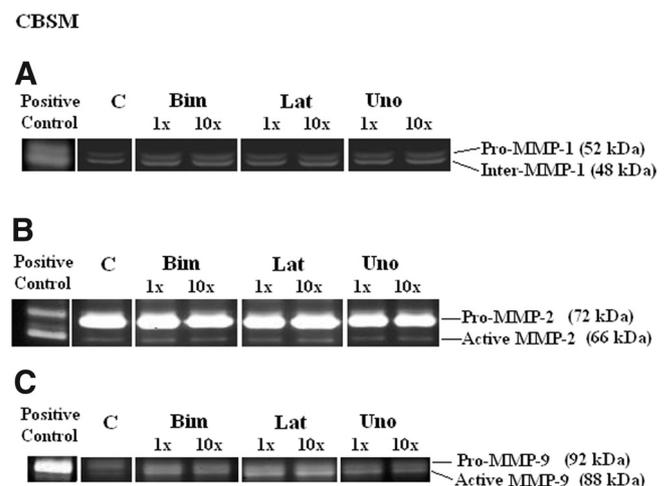


FIGURE 3. Representative zymograms for pro- and active forms of MMP-1 (A), -2 (B), and -9 (C) in human CBSM cells incubated with bimatoprost, latanoprost, and unoprostone for 24 hours. Active form of MMP-3 was not detected. C, control; 1× and 10× represent samples incubated at the peak aqueous concentrations and 10 times the peak aqueous concentration of these drugs, respectively, for 24 hours.

DISCUSSION

Our results indicate that the MMP and TIMP balance relates to the degree of IOP lowering exhibited by these three PGAs. All three agents lower IOP; the literature indicates that bimatoprost and latanoprost have at least an equivalent relationship with regard to lowering IOP.³⁸⁻⁴⁰ There are some studies that support a greater lowering of IOP by bimatoprost.^{38,39} The literature strongly supports a greater effectiveness of latanoprost compared with unoprostone.³¹⁻³⁷ Our findings implicate that the difference is due to a differential effect on TIMPs. Given the similar response of MMPs among the three PGAs, the different response of TIMPs would cause a different balance of MMPs and TIMPs. In general, MMPs are associated with increased ECM turnover. A higher proportion of TIMPs, the kinetic inhibitors of MMPs, may result in less ECM turnover and thus worse outflow facility through the uveoscleral pathway.

With latanoprost, we found a high correlation between MMP and TIMP protein expression and our previously published findings on mRNA levels in CBSM cells.¹² This finding is consistent with those in prior studies indicating that many MMPs are regulated at the level of transcription.⁴⁸ The exceptions were MMP-1, where the mRNA level was decreased whereas pro-MMP-1 protein expression was increased, and TIMP-1, where the mRNA was equivalent to baseline, but the protein level was increased. The reasons for the differences are unknown but may be the result of compensatory feedback inhibition.

The increase in pro-MMP-1, -3, -9 and the enzymatic activity of MMP-9 suggests a prominent role of these MMPs in PGA-mediated ECM turnover. Other groups have found increases in MMP-3 and -9 in response to latanoprost at the transcription, protein, and/or enzymatic levels.^{7,9,10,47} Notably, MMP-9 has been found diffusely distributed in ciliary body⁹ and mediates basement membrane degradation.⁴⁸ In contrast, we found that MMP-2 is unchanged by latanoprost or other PGA in transcription,¹² protein level, or enzymatic activity. However, the transcription and enzymatic activity of MMP-2 have been reported to be increased by latanoprost by other investigators.^{7,47} A limitation of our study of MMP activity is the absence of a detailed time course. The enzymatic activity may have been altered earlier than 24 hours. However, we found a very consistent response between the mRNA, protein, and kinetic activities of MMP-2 and -9. Gelatin zymography proved useful in the study of gelatinases, but casein had limited utility for collagenase-1 (MMP-1) and stromelysin-1 (MMP-3).

In patients and human tissue, variability of response between individuals is commonly noted with PGAs, both in clinical IOP response and the in vitro effect on MMPs, respectively. With regard to lowering IOP, the response rate (arbitrarily defined as >15% reduction from baseline) to latanoprost is approximately 85%.⁴⁹ However, among those who respond, the level of response varies. For example, Camras and Hedman⁴⁹ found the approximately 75% of patients had >20% IOP reduction but approximately 30% had >30% lowering of IOP. Bimatoprost has demonstrated similar variability of IOP reduction between individuals.⁵⁰ Similar to the clinical situation, our laboratory and others have noted variability in the response between cell lines from different donors in response to prostaglandins.^{7,12,46,47}

It has been reported that the size of pro-MMP-1 released by various human cell types ranges from 52 to 62 kDa, depending on glycosylation.^{7,51-53} The size of active MMP-1 has been reported to be between 41 to 47 kDa.⁵⁴⁻⁵⁷ We found two bands at approximately 52 and 48 kDa. Therefore, we postulate that the 48-kDa form is the intermediate form of MMP-1, similar to findings in human umbilical vein endothelial cells.⁵⁸ We could not detect the expression of active MMP-3 in control

and treated samples by zymography. Casein zymography has a detection limit at least two orders of magnitude lower than that of gelatin zymography.^{59,60} In addition, MMP-3 (stromelysin-1) does not have high affinity to casein, further limiting its detection by casein zymography.

With regard to TIMPs, we have reported that the amount of mRNA of TIMP-1 is unchanged in response to latanoprost, but in this present study, we found that the protein level decreased.¹² Our findings are in contrast to those of Anthony et al.¹¹ who found TIMP-1 to be increased both at the mRNA and protein levels. Our results agree with theirs on the effect on TIMP-2. TIMP-3 was increased by all three PGAs which is consistent with the increase at the mRNA level caused by latanoprost.¹² TIMP-3 has some unique qualities; it is the only TIMP that is sequestered in ECM to heparin-sulfate and chondroitin-sulfate containing proteoglycans.⁶¹⁻⁶⁵ TIMP-3 strongly inhibits not only MMPs but several ADAMs (a disintegrin and metalloproteinases), ADAMTS (ADAM with thrombospondin motifs), and tumor necrosis factor (TNF)- α -converting enzyme (TACE).⁶¹⁻⁶⁶ Upregulation of TIMP-4 by unoprostone other than bimatoprost and latanoprost is likely to mediate down-regulation of MMP-2 because TIMP-4 has a relatively higher potency of MMP-2 inhibition than other MMPs.⁶⁷

The exact mechanism of the relative differences in MMP/TIMP balance is unknown. It is possible that receptor binding affinity plays a role. Although there is disagreement on the relative binding affinity strength between bimatoprost and latanoprost, all studies show lower binding affinity of unoprostone to the FP receptor.^{27,29,30,68,69} In most tissues, the MMP/TIMP balance determines the rate of ECM turnover.^{15,19,21} Therefore, the decrease in MMP-2 and increases in TIMP-1 and -4 by unoprostone compared to latanoprost and bimatoprost may explain the lower clinical efficacy of unoprostone in lowering IOP compared with latanoprost and bimatoprost.^{34-37,40,70-76} Our findings may have some benefit in future drug development, as forthcoming PGAs may be screened inexpensively by testing their effects on MMPs and TIMPs.

Acknowledgments

The authors thank Kathryn A. Colby, MD, PhD, and Roberto Pineda, MD (both at Massachusetts Eye and Ear Infirmary), for the generous gifts of donor corneoscleral rims.

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