Published in final edited form as:

Am J Ophthalmol. 2008 January ; 145(1): 114–119. doi:10.1016/j.ajo.2007.09.001.

Prostaglandins Increase Trabecular Meshwork Outflow Facility in Cultured Human Anterior Segments

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Abstract

PURPOSE—To determine the effect of latanoprost free acid and prostaglandin E1 (PGE1) on outflow facility in cultured human anterior segments. Clinical studies find prostaglandin treatment increases uveoscleral outflow, but do not agree whether trabecular outflow increases. Cultured anterior segments eliminate the uveoscleral pathway and enable a direct assessment of trabecular outflow.

DESIGN—Laboratory investigation.

METHODS—One anterior segment of an eye pair was placed in perfusion organ culture and received a continuous infusion of drug while the fellow anterior segment received vehicle. Histologic changes were assessed. Zymography and Western blots were used to analyze matrix metalloprotease (MMP) activity. Scleral hydraulic conductivities were measured.

RESULTS—Latanoprost significantly increased outflow facility ($67\% \pm 11\%$ vs control $6\% \pm 10\%$, $P < .001$). Facility changes occurred within one hour of receiving drug, reaching a new baseline by 24 hours. Facility changes were reversible, requiring about 48 hours to return to pre-drug values. PGE1 caused less facility change ($13\% \pm 17\%$ vs control $1\% \pm 11\%$, $P = .02$). Prostaglandin treated anterior segments had regions of focal detachment and loss of Schlemm canal endothelial cells, with loss of extracellular matrix underlying some areas. MMPs were not consistently increased in Western blots, zymography, or immunohistochemistry. Scleral hydraulic conductivity increased, but not enough to account for total facility increase.

CONCLUSIONS—Prostaglandins increase outflow facility in perfusion organ culture of human anterior segments. MMP activity was not consistently increased, and scleral hydraulic conductivity was not increased sufficiently to account for total facility increase. The histologic changes suggest a direct trabecular meshwork effect.

Prostaglandin (pg) therapy lowers Intraocular pressure (IOP) in both glaucomatous and normal eyes. It induces cells of the ciliary body and sclera to increase their synthesis of matrix metalloproteases (MMPs).^{1,2} These changes fit clinical studies on the mechanism of PG action: increased uveoscleral outflow (“pressure insensitive” outflow).^{3–8} Clinical studies are conflicting whether PGs also affect conventional outflow facility (“pressure sensitive,” usually considered trabecular outflow), with some reporting an increase of 45%.^{5–8}

Although it is likely that an increase in conventional pressure-sensitive outflow facility would be from changes in the trabecular meshwork, an increase in scleral fluid flow (hydraulic

conductivity) could also play a role. Fluid flow through the sclera is known to be pressure-sensitive, with reports of hydraulic conductivity ranging from 0.003 to 0.020 $\mu\text{l}/\text{min}/\text{mm Hg}$ per cm^2 .^{9–10} PGs increase scleral permeability to solutes (dextran),¹¹ but their effect on pure fluid flow (hydraulic conductivity) has not been assessed. Although an increase in permeability to solutes is often accompanied by an increased hydraulic conductivity, this is not always the case.

This study looked at two prostaglandin analogues, latanoprost free acid and prostaglandin E1 (PGE1), and analyzed their effect on outflow facility. If pressure-sensitive outflow is increased, and the increase is from changes in the trabecular meshwork, PGs would represent the first “trabecular meshwork specific” drugs in addition to their role in the ciliary body. They would serve as prototypes for future drugs, and also indicate targets for future gene therapy.

METHODS

PERFUSION ORGAN CULTURE OF HUMAN ANTERIOR SEGMENTS

A total of 22 pairs of fresh normal human eye bank eyes were studied. Eyes were enucleated within 4 ± 2 hours postmortem and placed in culture within 11 ± 5 hours. The mean age of the donor eyes was 66 ± 14 years. No eyes had glaucoma, uveitis, pseudoexfoliation syndrome, or were on topical medications. The culture technique was similar to that described previously.^{12,13} Eyes were bisected at the equator, and the iris, lens, and vitreous were removed. The anterior segment was clamped in a modified petri dish and the anterior segment perfused with Dulbecco's modified Eagle's medium with added antibiotics (penicillin: 10,000 units, streptomycin: 10 mg, amphotericin B: 25 mg, and gentamicin: 17 mg, in 100 ml medium; Sigma, St Louis, Missouri, USA) at the normal human flow rate (2.5 $\mu\text{l}/\text{min}$). The anterior segments were cultured at 37 C in a 5% CO_2 atmosphere. IOPs were continuously monitored with a pressure transducer connected to the second access cannula built into the dish, and recorded with an automated computerized system. Attributable to the loss of choroid and ciliary body, this model does not have uveoscleral outflow, but does allow for measurement of the trabecular meshwork.

After an initial adaptation period in culture (generally one to three days), one anterior segment of a pair was given latanoprost free acid (100 nM, the amount of active compound present in the aqueous humor after cleavage of the ester group during drug entry¹⁴) or PGE1 (100 nM) via an anterior chamber exchange while the fellow control anterior segment received an anterior chamber exchange with culture media and an equal volume of ethanol (no drug). Following anterior chamber exchange, latanoprost, and PGE1 were continuously infused for up to 72 hours. Latanoprost free acid and PGE1 (Cayman Pharmaceuticals, Ann Arbor, Michigan, USA) were dissolved in 100% ethanol and stored at -20°C at 10^{-4} M concentration before final dilution and use. The anterior chamber exchanges were performed by a gravity-driven, constant pressure method over a five minute period. Pressure data from either anterior segment was not used during the first hour after the anterior chamber exchange.

Prostaglandin effects were expressed as the change in outflow facility (C) for each anterior segment.¹⁵ Results from each pair of anterior segments were combined into a group mean for each drug. Values are expressed as mean $\pm$ standard deviation. Statistical significance was analyzed using a two-tailed *t* test.

HISTOLOGIC EXAMINATION

At the end of the experiment, wedges of the limbus 180 degrees apart, including the trabecular meshwork, were isolated and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, while the majority of the meshwork was dissected from the anterior segment and frozen at -70°C .

Histologic examination with light microscopy was performed on all cultured anterior segments to assess the appearance of the trabecular cells, looking for evidence of toxicity. Tissue was embedded in JB4 plastic, cut 3 μ m thick, and stained with toluidine blue. Examination was performed in a masked fashion using pre-determined criteria including cell shape, nuclear shape, preservation of cellular and nuclear membranes, cell-cell attachments, cytoplasmic covering of trabecular lamellae, preservation of endothelial cell lining of Schlemm canal, and integrity of trabecular lamellae.¹³ Meshworks were considered normal if trabecular cell numbers were observed and cells remained in their usual position on the lamellae (subjective assessment), and no or minor disruption of the juxtacanalicular tissue and trabecular lamellae were seen. Transmission electron microscopy was performed on sections isolated from both anterior segments in seven of the 22 pairs of eyes, masking the observers (C.R.H. and D.H.J.) as to which specimens were experimental and control.

MATRIX METALLOPROTEINASE ANALYSIS

Zymography—Effluent medium was collected daily from prostaglandin-treated and control anterior segments. Effluent media volume was concentrated to within 10% of the lowest volume collected among all daily effluents from a given pair of anterior segments and stored at -70°C . To look for MMP 2, 3, and 9 activity, Zymography was performed by placing 20 μ l of concentrated effluent in Laemmli sample buffer (without β -mercaptoethanol) and separating proteins on a 10% gelatin gel (MMP-2, 9) or a 12.5% casein gel (MMP3; Bio-Rad, Hercules, California, USA). Gels were bathed in 2.5% Triton X-100 for 30 minutes at room temperature to renature proteins. Gels were placed in development solution (50 mM Tris, 200 mM NaCl, 5 mM CaCl_2 , 0.02% Brij-35, pH 7.5; Bio-Rad) and incubated overnight at 37°C . Gels were stained with 0.5% Coomassie Brilliant Blue R-250 (in 40% methanol/10% acetic acid; Bio-Rad) for one hour at room temperature and destained with 40% methanol/10% acetic acid until clear bands appeared.

Western Blots—Concentrated effluent from anterior segments (20 μ l) was placed in Laemmli buffer, boiled, and separated on 4% to 15% SDS-PAGE gradient gels (Bio-Rad). Gels were transferred to polyvinylidene difluoride membrane (Millipore, Billerica, Massachusetts, USA) in 1x transfer buffer (50 mM Tris, 384 mM glycine, 0.01% SDS, 20% methanol) and membranes blocked in 20 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween and 2% evaporated milk, and probed with antibodies to MMP-3 (Chemicon Int, Temecula, California, USA) and MMP-9 (Calbiochem, San Diego, California, USA). This was followed with either a secondary horseradish-peroxidaselinked anti-rabbit or anti-mouse Ig antibody (GE Healthcare, Piscataway, New Jersey, USA). Antibody:antigen complexes were detected using ECL Western blot signal detection reagent (GE Healthcare).

Immunohistochemistry—Two limbal tissue samples (180 degrees apart) were dissected from each anterior segment and fixed in 4% paraformaldehyde/0.1 M phosphate buffer pH 8.0, dehydrated in a graded series of ascending ethanol and embedded in paraffin. Sections 5 μ m thick were mounted on glass slides and baked at 60°C for two hours. Tissue sections were deparaffinized in xylene and rehydrated in a series of descending ethanol. Sections were incubated at 95°C in 1 mM EDTA (pH 8.0) and blocked in phosphate buffered-saline containing 3% bovine serum albumin and 0.1% Triton. Sections were incubated with rabbit MMP-3 antibodies. FITC-conjugated anti-rabbit Ig was used as secondary antibody (Molecular Probes, Eugene, Oregon, USA). Tissue was stained with DAPI to highlight the nuclei (0.25 $\mu\text{g/ml}$).

SCLERAL HYDRAULIC CONDUCTIVITY

The method of Jackson and associates was used to assess scleral hydraulic conductivity.¹⁰ Scleral pieces 8 mm in diameter were isolated from prostaglandin-treated and control anterior segments following perfusion organ culture using a corneal punch (Acuderm, Inc, Fort

Lauderdale, Florida, USA). The scleral pieces were clamped in Ussing chambers (4 mm aperture; World Precision Instruments Inc, Sarasota, Florida, USA) in a vertical rather than horizontal orientation.¹⁰ Two scleral pieces from each anterior segment were analyzed simultaneously (Four Ussing chambers were used; $n = 6$ pairs of anterior segment). Phosphate-buffered saline (Mediatech, Herndon, Virginia, USA) with latanoprost free acid (100 nM) was added to each side of the Ussing chamber holding the prostaglandin-treated scleral button while phosphate buffer alone was added to both sides of the control scleral buttons. All scleral pieces were perfused in the “correct” anatomic direction, with the pressure head facing the choroidal side of the sclera to mimic physiologic trans-scleral flow. As described by Jackson,¹⁰ a 1 mm pipette was filled with saline and placed on the “upstream,” or choroidal side of the sclera. This resulted in a perfusion pressure of 12 mm Hg. Evaporation was measured using a filled pipette closed at the bottom and not connected to the Ussing chambers. The “downstream” chamber, receiving the perfusate after it had crossed the sclera, had an opening at the top of the chamber allowing fluid to exit the chamber as needed. The perfusion period was 24 hours. Scleral thickness was measured before and after placement in the Ussing chamber with a System Corneo-Gage II ultrasonic pachymeter (Sonogage, Cleveland, Ohio, USA).

RESULTS

OUTFLOW FACILITY

Latanoprost free acid significantly increased outflow facility ($67\% \pm 11\%$ vs control anterior segment $6\% \pm 10\%$, at 24 hours treatment; $P < .001$). PGE1 caused less increase in facility ($13\% \pm 17\%$ vs control anterior segment $1\% \pm 11\%$, $P < .02$). Latanoprost free acid had a significantly greater effect at each timepoint (Table). For both prostaglandins, the increase in facility began one hour after receiving the drug and continued to increase over the first 12 hours (Figure 1). In the latanoprost free acid group, a stable baseline was reached over the next 12 hours in eight of 15 anterior segments, while in the other seven anterior segments, facility continued to increase. At this point, after 24 hours of prostaglandin infusion, anterior segments were divided into two groups. In group 1, prostaglandin infusion was stopped to see if the drug effect was reversible, while group 2 had continued perfusion of the prostaglandin for a total of three days. In group 1, the latanoprost free acid effect was reversible, as outflow facility returned to original baseline levels over the next 19 ± 17 hours (Figure 1). If latanoprost free acid infusion was longer than 24 hours, however, the effect was not reversible (data not shown). In group 2, the continued prostaglandin infusion caused outflow facility to slowly increase during the entire three days, reaching a mean increase of $175\% \pm 105\%$ compared with fellow control anterior segments ($n = 8$; $P = .04$).

Histologic Examination—Both latanoprost free acid and PGE1 eyes had scattered focal region loss of Schlemm canal endothelial cells when compared with fellow control anterior segments (Figure 2). The cell loss did not appear to be a toxic effect, as cells of the corneoscleral and other regions appeared normal and were similar between prostaglandin-treated and control anterior segments. Instead, the inner wall cells of the canal were lifted off the basal lamina, consistent with loosening of the focal adhesions between cell and extracellular matrix.¹⁶ In two of the 14 eyes examined by transmission electron microscopy this local inner wall cell loss was accompanied by focal loss of underlying extracellular matrix of the juxtacanalicular region. Both of these anterior segments had received prostaglandins (one latanoprost; one PGE1).

Matrix Metalloproteinase Analysis—Analysis of effluent media from prostaglandin-treated anterior segments did not show a consistent change in MMP-2, 3, and nine activity or qualitative levels when compared with control anterior segments with either Zymography or

Western blots (data not shown). Only one of 10 pairs of anterior segments had an increase on Western blot and Zymography for MMP-3.

The effluent media collected from anterior segment culture may contain MMPs secreted from other anterior segment tissue (cornea, sclera, and ciliary body). Therefore, the possibility exists that slight increases in MMP levels within the trabecular meshwork following prostaglandin treatment may be diluted. To specifically analyze the level of MMP-3 in the trabecular meshwork, we performed immunohistochemistry. Analysis of six independent meshworks showed no definitive changes in MMP-3 activity.

Scleral Hydraulic Conductivity—Hydraulic conductivity was not significantly increased by latanoprost free acid treatment when compared with fellow control anterior segment sclera (0.055 ± 0.023 vs 0.034 ± 0.024 $\mu\text{l}/\text{min}/\text{mm Hg}$; $P = .16$; converted to these units to allow comparison with facility values). The small increase in scleral hydraulic conductivity (though not statistically significant) is not enough to account for total increase in the outflow facility taking into account the small baseline hydraulic conductivity of the sclera.^{9,10} Anterior scleral thickness was similar between experimental and fellow control anterior segments (0.64 ± 0.09 μm vs 0.67 ± 0.11 μm).

DISCUSSION

Prostaglandins increase outflow facility in the cultured human anterior segment. The increase in outflow facility began within several hours and the greatest change occurred within the first 12 hours during treatment. The effect was reversible if the prostaglandin was stopped after 24 hours, with facility returning to near the original baseline within a 48 hour period. If prostaglandin infusion was longer than 24 hours, the effect was not reversible. Of particular interest was the rapid increase in facility, noted as soon as two hours after drug infusion. Although most clinical studies report IOP changes 12 or more hours after receiving prostaglandins, several have examined IOP earlier and found significantly lower IOP as early as one to three hours in normal human eyes^{17–19} and two hours in monkey eyes.²⁰ The rapid change suggests that a mechanism in addition to induction of enzymes may be involved.

Previous studies report a direct effect of prostaglandins on the trabecular meshwork.^{21,22} Prostaglandin receptors are present in the trabecular meshwork and stimulation activates intracellular signaling: inositol phosphates and calcium mobilization.²¹ Dijkstra and associates found infusion of PGE1 for one hour in cultured human eyes increased outflow facility 24%. This was accompanied by a four-fold increase in cAMP.²¹ Latanoprost free acid treatment of isolated trabecular meshwork strips or cultured cells increases mRNA for MMP 1, 2, 17, and 24.²² In addition, prostaglandins relax contractility in bovine meshwork,²³ which may lower intraocular pressure. Overall, prostaglandins cause disassembly of actin stress fibers and inhibition of phosphorylation of paxillin and other focal adhesion proteins.¹⁶ These cytoskeletal and focal adhesion changes may loosen the attachment of Schlemm canal cells, causing focal cell loss. Schlemm canal cells would be less able to resist the perfusion pressure gradient between IOP and the lower pressure in Schlemm canal.²⁴ Thus several non-MMP events could explain the immediate drop in IOP we have found in cultured anterior segments.

In our study, histologic findings were consistent with these changes, as focal loss of Schlemm canal cells and loss of the underlying extracellular matrix in the juxtacanalicular region were observed. The trabecular meshwork cells in the uveal and corneoscleral regions appeared normal, with no appearance of toxicity or loss of their normal horizontal position on the lamellae. These changes are similar to findings in monkeys.^{22,25}

Direct evidence for changes in selective matrix metalloprotease enzymes in the trabecular meshwork was not found. Effluent medium collected at the limbus did not show changes in MMP 2, 3, and 9 profiles. It must be remembered that the contribution of proteins from the trabecular meshwork may be diluted or overwhelmed by proteins from the remainder of the anterior segment. All the other ocular structures are bathed in the same media, which ultimately exits through the trabecular meshwork and would be collected at the scleral surface after exiting the scleral veins. Thus, if the cornea, sclera, and remnant of ciliary body were also secreting MMPs 2, 3, and 9, these would obscure any subtle increases of the trabecular meshwork. In addition, immunohistochemistry, which provides direct evidence of MMPs in the meshwork, did not show an increase in MMP 3.

Another route for fluid exit from the eye in a pressure-dependent fashion could be through the sclera. Scleral fluid flow (hydraulic conductivity, analogous to outflow facility) is low, with values between 0.008 and 0.060 $\mu\text{l}/\text{min}/\text{mm Hg}$ (calculated from authors' data for the scleral area of our cultured anterior segments, 3.15 cm^2).^{9,10} This is much lower than outflow facility of the control anterior segments in our study ($0.21 \pm 0.08 \mu\text{l}/\text{min}/\text{mm Hg}$) indicating only a small portion of fluid exits the sclera in this culture model. Prostaglandins caused a tendency to increase scleral hydraulic conductivity in these cultured anterior segments, but the difference was not statistically significant and was small enough that it would not explain the total increase in outflow facility. To account for the prostaglandin-induced increase in outflow facility, scleral hydraulic conductivity would need to have increased about 300%. Prostaglandins increase scleral permeability to dextran in human sclera and whole enucleated mouse eyes.^{26,27} Hydraulic conductivity to water, however, differs from permeability to solutes such as dextran, preventing quantitative comparisons.

In conclusion, this study supports the clinical findings of a prostaglandin-induced increase in outflow facility in the human eye.^{5–8} Because this culture model of the human anterior segment does not have a choroid or functional ciliary body, the uveoscleral pathway does not exist. This simplifies the analysis, allowing a direct measurement of the contribution of the sclera to outflow facility. The scleral role is small, indicating prostaglandins increase flow through the trabecular meshwork.^{5–8}

Acknowledgments

This study was supported by National Institutes of Health, Bethesda, Maryland, Research Grants EY 07065 and EY15736; Research to Prevent Blindness, Inc, New York, New York; the Philanthropy of The Bonner Glaucoma Research Fund, Rochester, Minnesota; and Mayo Foundation, Rochester, Minnesota. The authors indicate no financial conflict of interest. Involved in design and conduct of study (M.P.F., D.H.J.); data collection (C.K.B., K.G.H., C.R.H.); management, analysis, and interpretation of data (C.K.B., K.G.H., C.R.H., M.P.F., D.H.J.); preparation of manuscript (D.H.J.); review of manuscript (C.K.B., K.G.H., C.R.H., M.P.F.); and approval of manuscript (C.K.B., K.G.H., C.R.H., M.P.F., D.H.J.). The Mayo Clinic College of Medicine Institutional Review Board approved the use of human autopsy eyes for this study.

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FIGURE 1.

Reversibility of latanaprost free acid treatment on intraocular pressure (IOP) in human cultured anterior segments. Latanaprost free acid was given for 24 hours causing a prompt decrease in pressure. After removal of latanaprost free acid, pressure returned to baseline over 48 hour period.

FIGURE 2.

Prostaglandin-E1 (PGE1) effect on human trabecular meshwork. (Top) Control anterior segment (an 86-year-old female). Outflow facility remained stable during culture. Schlemm canal cells are intact (arrowheads). Trabecular cells have normal nucleoli and position on lamellae (600 $\times$). (Bottom) Fellow anterior segment that received PGE1. Outflow facility increased 54% over 72 hours, with IOP dropping from 20 mm Hg to 14 mm Hg. Schlemm canal cells show intercellular separations (arrows) and loosening from underlying extracellular matrix (asterisks). Trabecular cells are intact, with normal nuclei and position on lamellae. SC = Schlemm canal (600 $\times$).

TABLE 1
Effect of Prostaglandins on Outflow Facility in Human Eyes

	n	Eye Pair	Baseline	12 hr	24 hr	48 hr	72 hr
Prostaglandin-E1	7	expt	0.15 ± 0.05	0.19* ± 0.04	0.20* ± 0.03	0.20* ± 0.05	0.22* ± 0.09
		con	0.17 ± 0.07	0.19 ± 0.09	0.19 ± 0.10	0.18 ± 0.08	0.18 ± 0.08
Latanoprost (continuous drug)	8	expt	0.15 ± 0.03	0.24* ± 0.09	0.24* ± 0.09	0.34* ± 0.19	0.40* ± 0.25
		con	0.18 ± 0.05	0.18 ± 0.05	0.19 ± 0.06	0.19 ± 0.05	0.21 ± 0.06
Latanoprost reversibility (drug stopped after 24 hr)	7	expt	0.12 ± 0.03	0.19* ± 0.05	0.20* ± 0.06	no drug	no drug
		con	0.25 ± 0.08	0.24 ± 0.07	0.24 ± 0.07	ND	0.13 ± 0.03
						ND	0.24 ± 0.08

Hr = hours; ND = not determined; expt = experiment; con = control.

Facility of outflow: ul/min/mm Hg expressed as mean ± standard deviation.

* $P < .05$.