FROM THE BEDSIDE TO THE BENCH AND BACK AGAIN: PREDICTING AND IMPROVING THE OUTCOMES OF SLT GLAUCOMA THERAPY

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ABSTRACT

Purpose: To determine whether selective laser trabeculoplasty (SLT) and prostaglandin analogues (PGAs) have a common mechanism of action that involves increasing conductivity across Schlemm's canal endothelial cells (SCEs) and inducing a similar decrease in intraocular pressure (IOP) in a given patient.

Methods: The intercellular junctions in SCEs were made visible by transfection of a plasmid containing a GFP-tagged gene for ZO-1 protein. Transfected SCEs were treated with media conditioned by lasered trabecular meshwork endothelial cells (TMEs), or with latanoprost, bimatoprost, or travoprost. Non-transfected SCEs were exposed to brimonidine, timolol, or brinzolamide. Confocal microscopy and conductivity measurements documented the in vitro treatment effects. Clinically, the IOP in the first SLT-treated eye of 24 patients was measured (1) while on PGA therapy, (2) at "baseline" several weeks after discontinuing PGA therapy, and (3) ~90 days after SLT treatment.

Results: Both the in vitro addition of any of the 3 PGAs and of media conditioned by lasered TMEs induced similar SCE effects involving junction disassembly, paracellular pathway widening, and increased conductivity. Clinically, PGAs decreased IOP by a mean of 5.58 mmHg and SLT decreased IOP by 6.60 mmHg from a baseline of 21.52 mmHg.

Conclusions: Exposure to media conditioned by lasered TMEs, or the addition of PGAs, induces the disassembly of intercellular junctions opening up the SCE barrier. Clinically, a positive PGA response predicts both a successful SLT outcome and the magnitude of the decrease in IOP after SLT. We hypothesize that SLT and PGA therapies may share a common mechanism of action.

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INTRODUCTION

"At the bedside," while managing patients treated with selective laser trabeculoplasty (SLT), we were perplexed by the low success rate and modest intraocular pressure (IOP)–lowering effect achieved in some patients. Several possible explanations were considered for this unsatisfactory but relatively common outcome.[1] As a tertiary care referral center, our glaucoma patient population included many individuals with a particularly severe disease process. Most patients were already taking multiple topical glaucoma medications and had suffered significant visual field loss and cupping of the optic nerve. Another explanation was that the baseline IOP in our patients was relatively low as their glaucoma was managed aggressively with multiple topical medications. Low baseline pressures have been associated with lower success rates and a reduced pressure-lowering effect.[1-4] Despite these and other possibilities, the hypothesis we were more interested in testing was that the most potent topical glaucoma medications in current use interfere with the success of the laser procedure by competing for a common mechanism of action, and were responsible for our relatively disappointing SLT outcomes.

The 6 topical medications in common use by our patients included brimonidine, timolol, and brinzolamide and 3 prostaglandin analogues (PGAs)—latanoprost, bimatoprost, and travoprost. The non-PGA topical medications were considered unlikely SLT competitors as they function by decreasing aqueous production,[5] which is presumably not affected by laser trabeculoplasty. On the other hand, the PGAs were likely to interfere with SLT, as they are outflow agents,[6,7] although the precise mechanism of action is currently under investigation. In testing our hypothesis we were guided by studies conducted in our laboratory over 2 decades ago, which showed that adding prostaglandins onto cultured trabecular meshwork endothelial cells (TMEs) increased flow across these cells (unpublished observations). Guidance was provided as well by a historical fact indicating that the use of argon laser trabeculoplasty (ALT) declined in a close temporal relationship with the introduction and rapid acceleration in the utilization of PGAs for glaucoma therapy.[8] We wondered if perhaps this decline in the ALT utilization in the 1990s was related to the occurrence of progressively unimpressive outcomes due to competition for the same mechanism of action between PGAs and ALT, much as we are proposing is taking place today between PGAs and SLT. Alternatively, the decline in ALT may have been related to the high efficacy of PGAs making fewer patients "candidates" for ALT.

We went to "the bench" to learn whether there were any competitive interactions between PGAs and SLT affecting the integrity and hydrodynamic properties of the fluid barrier formed by Schlemm's canal endothelial cells (SCEs). A technique was implemented to visualize the intercellular junctions forming the SCE barrier under "living" conditions so that we could correlate the junction assembly/disassembly process with dynamic permeability changes affecting the barrier after laser irradiation and after the addition of topical glaucoma medications using in vitro conditions. The addition of media conditioned by lasered TMEs or the direct addition of PGAs onto SCE monolayers resulted, in both circumstances, in the disassembly of junctions making up the SCE barrier and in increased permeability. Upon learning "at the bench" that our hypothesis might be correct, we went back to the clinical setting to learn whether removal of PGAs prior to SLT was associated with improved outcomes, defined as an increased success rate and greater IOP-lowering effect.

We have now adopted a new SLT treatment protocol in our clinic where PGAs are discontinued several weeks prior to SLT. This

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study reports the outcome of this new protocol in a cohort of 24 consecutive patients treated by SLT. All patients experienced an IOP increase following the removal of PGAs, and this IOP increase predicted both a positive response to the SLT and the magnitude of the response. Our results also suggested an improved SLT response with removal of PGAs, which provides further support for the validity of our laboratory studies.

METHODS

The aim of our research project required that we carry out 2 studies involving the use of 2 very different approaches. The first study involves in vitro experiments assessing the effect of laser irradiation and various topical glaucoma medications on SCEs. To keep the goal of each study clearly in mind, the assays for these in vitro experiments are described separately under the heading "Laboratory Studies" below. The second part of our investigation is directed at finding out whether removing PGAs in a given patient prior to delivering SLT has an impact on the pressure-lowering effect of this laser irradiation. The methods used in the second part are described separately under the heading "Clinical Studies" below.

METHODS: LABORATORY STUDIES

In the first set of laboratory experiments, we examined the effects associated with the addition of media conditioned by laser-irradiated TMEs onto SCEs. The second set of in vitro experiments included adding separately 1 of 6 topical medications commonly used today to treat glaucoma. The effects of both sets of experiments on SCEs were evaluated in terms of the intercellular junction disassembly process and associated permeability effects. The SCE-barrier effects were examined using confocal microscopy and immunofluorescent methods. The conductivity effects were measured using sensitive flow meters.

Cell Culture and Transfection to Visualize Intercellular Junctions

Primary human cultures were established as previously described for SCEs and TMEs.[9-12] Human explants of SCEs and TMEs were grown separately in collagen IV–coated 100-mm culture dishes (BD, Franklin Lakes, New Jersey) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 2 mM L-glutamine, 50 µg/mL of gentamicin, and 2.5 µg/mL amphotericin B (all from Mediatech, Manassas, Virginia). For permeability studies, SCEs were grown in collagen IV–coated Millipore filter supports (Millipore, Billerica, Massachusetts). Madin-Darby canine kidney (MDCK) epithelial cells were grown using similar methods and used as controls.

The intercellular junctions were made visible by transfecting cells with a plasmid construct, pEGFP-C1 ZO-1, expressing zonula occludens-1 (ZO-1) protein tagged with green-fluorescent protein (GFP) (GFP-ZO-1) as previously described.[13-15] Plasmid amplification and purification were carried out using EndoFree Plasmid Maxi Kit (Qiagen, Valencia, California) following the manufacturer's specifications. The plasmid purity was confirmed using restriction digestion with XhoI (New England BioLabs, Inc, Ipswich, Massachusetts) and gel electrophoresis to ascertain the presence of 3.5 and 8 kilobase (kb) DNA bands. We used the nucleofection approach to incorporate the plasmid vector into our cultured cells.[16]

For the transfection experiments, confluent fifth passage SCEs, TMEs, and MDCK cells were dissociated using 0.05% trypsin and pelleted by centrifugation at 100×*g* for 10 minutes at room temperature. After aspiration of the media, the cell pellet was resuspended in Basic Nucleofector Solution (Lonza, Basel, Switzerland) to a concentration of 5×10^5 cells/100 µL. The resuspended cells underwent nucleofection using the Lonza nucleofector (Lonza, Basel, Switzerland) set to the program #T-27 for each transfection reaction. The cells were transfected using 3 µg of the purified plasmid, and transfected cells were immediately seeded on 4-well Lab-Tek chamber slides (Nunc/Thermo Fisher Scientific, Rochester, New York) at a density of 2×10^5 cells per well and maintained in the standard media described above. The preps were incubated for 48 hours in a humidified incubator set at 37°C in 8% CO_2 just prior to conducting the experiments described below.

Treatment Protocols for the Cultured SCE Preparations

The assembly/disassembly of intercellular junctions in SCEs, and associated conductivity effects, were examined following a wide variety of treatments, including laser irradiation, and exposure to 6 topical glaucoma medications. The frequency-doubled, Q-switched neodymium YAG (F-D Nd:YAG) laser was used to irradiate human cultured TMEs and SCEs. To mimic the situation encountered during SLT in patients, a number of cells roughly similar to those present in the trabecular meshwork in vivo were irradiated using the F-D Nd:YAG laser. Thus, one million TME cells received ~100 laser applications measuring 400 µm in diameter, and 0.66 mJ in power. Media conditioned by the laser-irradiated TMEs was then collected and added to untreated SCEs as previously described.[12,17] Fresh bottles of 3 commercially available topical PGA-based glaucoma medications in current clinical use were tested: latanoprost (Pfizer/Pharmacia & Upjohn Company, Kalamazoo, Michigan), travoprost (Alcon Laboratories, Inc, Fort Worth, Texas), and bimatoprost (Allergan, Inc, Irvine, California). The 3 PGAs were prepared for addition to the cultured SCEs by adding one drop of each medication (with each drop measuring about 30 µL), after shaking the bottle for several minutes, to 1 mL of cell-culture media. The final concentrations were approximately 3.5 µM for latanoprost, 2.4 µM for travoprost, and 20 µM for bimatoprost. Similarly, we prepared dilutions of brimonidine (Allergan, Inc, Irvine, California), timolol (Merck & Co., Inc, Whitehouse Station, New Jersey), or brinzolamide (Alcon Laboratories, Inc, Fort Worth, Texas) by adding one drop of each medication, obtained from freshly opened bottles, to 1 mL of freshly prepared culture media, yielding a concentration of ~0.14 mM for brimonidine, ~0.39 mM for timolol, and ~0.78 mM for brinzolamide. SCEs plated on Lab-Tek chamber slides received 700 µL of the diluted medication. In addition, ethacrynic acid (Sigma-Aldrich, St Louis, Missouri) was added to SCEs to examine the intercellular junction assembly/disassembly

process. Ethacrynic acid was freshly prepared as a 100 mM stock solution in 50% ethanol, which was then further diluted to 0.8 mM in culture media immediately before addition to the cultured SCEs.

Examination of the Treatment Effects on the SCE Preparations

The junction assembly/disassembly process was examined in both transfected and nontransfected cells using 3 different techniques, including confocal microscopy, immunofluorescence, and flow meters to measure permeability effects in the variously treated cultured SCEs.

Confocal Laser Scanning Microscopy. The junction assembly/disassembly in the transfected cells was examined in "real time" using the Zeiss LSM 5 Pascal confocal microscope (Argon laser 488 nm and HeNe laser 543 nm; Carl Zeiss, Inc, Thornwood, New York). The preps were maintained at 37°C with 5% CO_2 during the time-lapse photography sessions, which lasted for approximately 5 to 30 seconds. During these sessions, the focus in the confocal microscope was adjusted manually without changing brightness and contrast. The images obtained were further processed using the ImageJ software (National Institutes of Health, Bethesda, Maryland, http://rsb.info.nih.gov/ij/) and the LSM Image Browser (Carl Zeiss, Inc, Thornwood, New York) to improve image quality and to provide animation to the sequence of the numerous still photographs acquired.

Immunofluorescence Assays. Nontransfected cells were plated on Lab-Tek chamber slides and examined using immunofluorescence methods to ascertain that findings acquired using transfected cells were verified with nontransfected SCEs. Treated or control cells were labeled immunologically, as described previously,[9,18] by sequential incubation with anti-ZO-1 antibody, biotin-conjugated secondary antibody, and Cy3-streptavidin. Immunologically stained preparations were observed under the confocal fluorescent microscope to document the intercellular junction status in the variously treated SCEs.

Conductivity Measurements. The conductivity of cell monolayers was assessed using sensitive flow meters and a computer-driven hydraulic conductivity apparatus previously described.[12,17-19] Briefly, cultured cells grown over porous filter supports were kept in a humidified 8% CO_2 incubator and fed every 48 hours with media containing 15% FBS until they reached confluence (about 10 to 14 days), and the FBS concentration was then reduced from 15% to 10%. These preparations were then exposed to experimental and control conditions, and the conductivity was measured in μL/min/mm Hg/cm^2 at a constant pressure of 4.5 mm Hg.

METHODS: CLINICAL STUDIES

During the summer of 2008 we implemented a new SLT-treatment protocol where PGA medications were removed several weeks prior to the laser procedure. For this study, we reviewed retrospectively the charts of the first 24 consecutive primary open-angle glaucoma patients treated with this new SLT protocol by a single provider (J.A.A.). Only the first eye treated per patient was analyzed in cases of bilateral SLT treatment. Three IOP measurements were recorded. The first measurement was recorded while the patient was being treated with a PGA along with other glaucoma medications. The PGA was then discontinued, and several weeks later, immediately before the SLT procedure, the second IOP measurement was recorded—a measurement taken to represent the "baseline" IOP. Finally, ~90 days after the SLT treatment, the IOP was measured for the third time—a measurement taken to represent the IOP response to the SLT procedure. Exclusion criteria included patients younger than 18 years of age, patients who were "status post incisional glaucoma surgery" in the eye being studied, prior or subsequent corneal transplants, and less than 3 months of follow-up. All patients included in the study had 360° SLT treatment. Patients with prior ALT treatment were included in the analysis.

RESULTS

TRANSFECTION WITH A CONSTRUCT CONTAINING A GENE FOR GFP-TAGGED ZO-1

SCEs and MDCK cells transfected with the GFP-ZO-1 construct vigorously synthesize the GFP-tagged ZO-1 protein so that only 48 hours after transfection there was intense fluorescence along the cell margins (Figure 1), making the intercellular junctions readily visible under the fluorescent and confocal microscopes. In Figure 1 we contrast the appearance of the intercellular junctions in MDCK cells, representing the arrangement typically observed in most epithelial and vascular endothelial cells, with the unique appearance of the junctions in SCEs. Note that in MDCK cells, the junction proteins are located strictly within the paracellular route assuming a linear arrangement, looking much like the caulking that separates tiles in a mosaic (left panel). In a sharp contrast and as we have previously described using standard immunofluorescence studies and transmission and scanning electron microscopy,[9,18] the junction proteins in SCEs differ in two important respects. First, unlike in MDCK cells, the junction proteins in SCEs are not confined to the paracellular route per se, but instead these proteins are deposited along the apical borders and atop the apical surface of neighboring cells. Moreover, the proteins are deposited along fine finger-like structures that extend from the apical most boundary of one cell, across the intercellular space, and onto the apical surface of adjacent neighboring cells. These so-called filopodia have the underside resting atop the apical cell surface, and both apposing surfaces are densely coated with junction proteins, which effectively bind each filopodium to the underlying cell surface. In cultured SCEs, the finger-like structures form a palisade arrangement, which is made up of multiple filopodia disposed in parallel to each other (right panel). In this manner, filopodia bind neighboring cells firmly to each other, much as fingers bind hands that are tightly clasped together. Similar features were observed in cultured TMEs (data not shown).

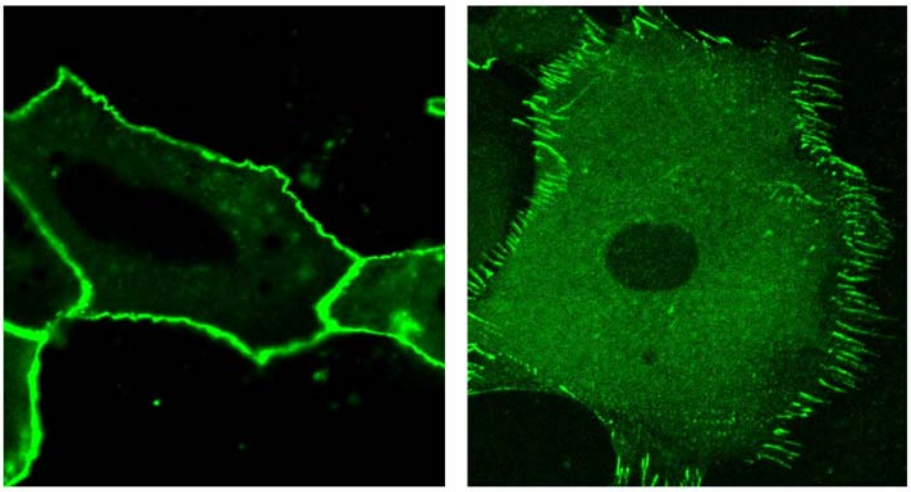

FIGURE 1

Comparison of the intercellular junction appearance in Madin-Darby canine kidney (MDCK) (left panel) and Schlemm canal endothelial (SCE) cells (right panel). Both cell types were transiently transfected with a plasmid construct coding for the junctional protein ZO-1 tagged with GFP (GFP-ZO1). Note that in MDCK cells the GFP-ZO1 is deposited in a linear manner along the paracellular route, whereas in SCEs the GFP-ZO1 is deposited along filopodia (original magnification ×40).

THE JUNCTION DISASSEMBLY/ASSEMBLY PROCESS IN MDCK CELLS

The intercellular junction assembly and disassembly process in the MDCK cells was photographed using time-lapse confocal microscopy, from which we selected snapshots illustrating various stages in this process. In Figure 2 (upper left panel), the assembled junctions between 2 cells are shown at time zero, before adding calcium-free media to these cells. The removal of calcium inhibits the protein kinase C pathway inducing actin depolymerization and contraction of the cytoskeleton with the concurrent widening of the intercellular space.[13,20,21] As shown in the upper right panel of Figure 2, taken 32 minutes after calcium removal, the intercellular junctions remain stretched but still assembled, although the paracellular route is clearly widened. In fact, this space is filled with stretched fibrils that do stain for ZO-1. Also note some fine fibrils in the cytoplasm that are connected to the cell margin and the ZO-1 protein. These green fibrils are straightened out, suggesting contraction and the presence of ZO-1 in association with the fibrillar cytoskeletal system. ZO-1 has been recently shown to be associated with actin filaments in both a cytoplasmic location and at the cell membrane as a "cytoplasmic accessory protein."[13,21,22]

In Figure 2 (middle left panel), the intercellular junction has undergone disassembly 50 minutes after the addition of the calcium-free media. The 2 neighboring cells are now separated by a widened space, which is free of any assembled intercellular junctions. The cells themselves, presumably due to contraction of the actin cytoskeleton, are rounded and smaller in appearance. At the end of the junction disassembly process, the GFP-tagged ZO-1 protein is easily visible in the inner aspect of the cell membrane, forming a prominent circular ring of brightly fluorescent ZO-1 protein. This finding is consistent with the mentioned localization of ZO-1 to the inner aspect of the cell membrane where this protein is linked to the cytoskeleton.[13,15] Therefore, the "linearization" of the ZO-1 protein complex in this experiment points to the conclusion of the junction disassembly process. This linearization is also observed in the case of SCEs (and TMEs) as the intercellular junctions become fully disassembled.

The middle right and both lower panels of Figure 2 depict the junction-assembly process in cells exposed to a calcium-enriched media. At time zero, in the middle right panel of Figure 2, 2 cells are shown separated by a widened space containing no intercellular junctions. At 32 minutes after the addition of 3.6 mM of calcium, shown in the lower left panel of Figure 2, the intercellular space is clearly narrowed, as the cytoplasm undergoes relaxation, and contact is made in the one location where these 2 cells were formerly bound to each other at the beginning of these experiments, much in the same fashion as seen in the upper left panel of Figure 2. Note the bright green fluorescence at the point of contact which may signify the initiation of the cell junction assembly process. At 70 minutes, the lower right panel of Figure 2, the region of bright green fluorescence is extended to cover an enlarging area of apposition between the 2 neighboring cells, and the appearance of this region is very much reminiscent of that shown in the upper left panel of Figure 2, where the junctions are fully assembled.

JUNCTION DISASSEMBLY PROCESS IN SCES

SCEs transfected with the GFP-ZO-1 construct were exposed to 0.8 mM of ethacrynic acid diluted in standard culture media while the junction disassembly process was continuously monitored using time-lapse confocal microscopy. In Figure 3 we illustrate the entire

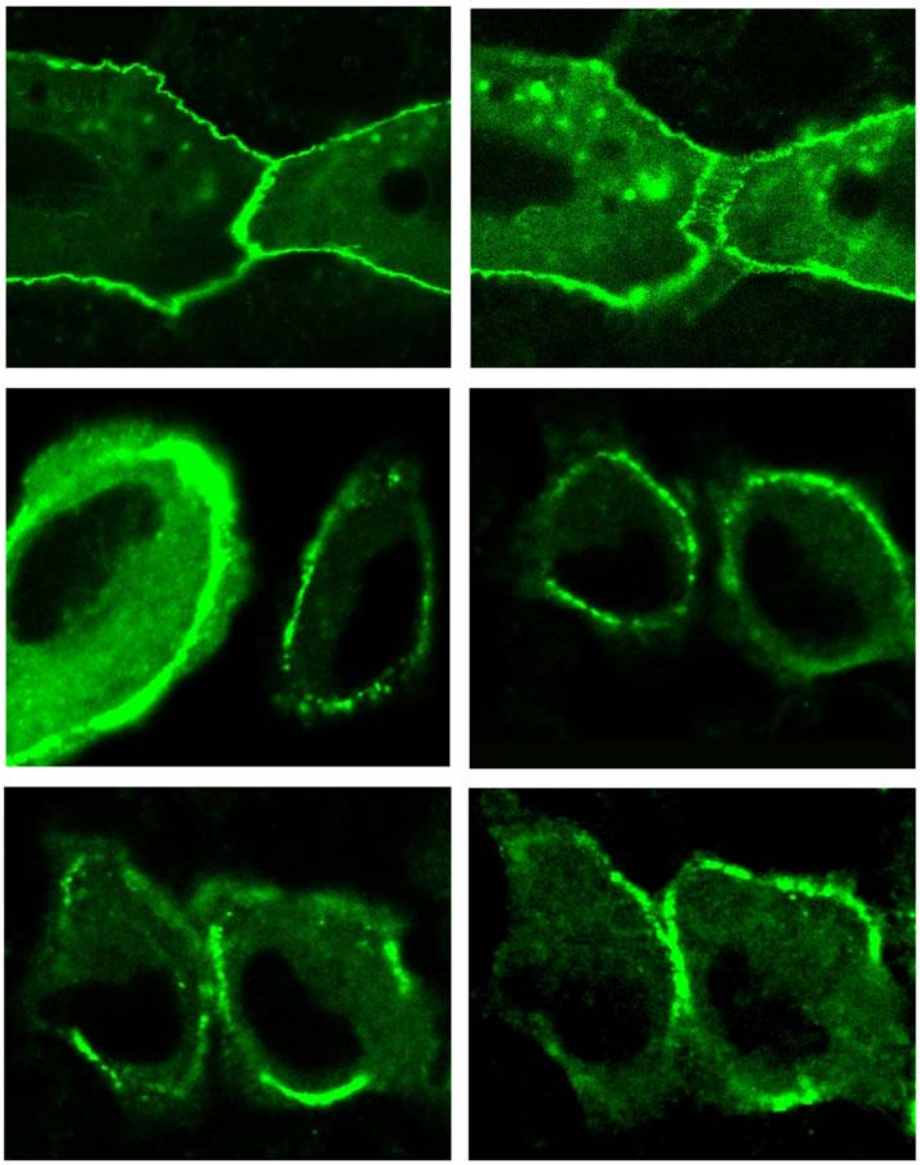

FIGURE 2

Snapshots showing the junction disassembly and assembly process in Madin-Darby canine kidney cells (MDCKs). Time-lapse confocal microscopy was used to document the junctional disassembly process using Ca-free media conditions for 1 hour, and the assembly process was documented after the addition of calcium. Snapshots taken at 0 minutes (upper left), at 32 minutes (upper right), and at 50 minutes (middle left) illustrate the junctional disassembly process. Snapshots taken at 0 minutes (middle right), at 32 minutes (lower left), and at 70 minutes (lower right) document the junctional assembly process. (original magnification ×63).

junction disassembly process in several snapshots taken at the indicated times. Ethacrynic acid interacts with actin and other components of the cytoskeletal apparatus and induces cellular contraction.[23-26] The net ethacrynic acid effect is to induce the separation of adjacent cells, which is opposed by the intercellular junctions, until the latter's integrity is disrupted and their disassembly takes place rapidly. The upper left panel of Figure 3 shows that at the outset of the experiment, numerous filopodia bind 2 neighboring cells onto each other. Twenty-two minutes later (upper right panel of Figure 3), as the intercellular space begins to widen (asterisk), the filopodia become stretched with contraction of the cytoskeleton under the influence of ethacrynic acid. This process progresses rapidly, so that at 32 minutes (lower left panel of Figure 3), only some of the filopodia span across the width of the further widened intercellular space, while many have undergone complete retraction and shortening.

The disassembly process is nearly completed at 37 minutes (lower right panel of Figure 3), when the intercellular space has widened 5- to 10-fold (asterisk), allowing visualization of the basal margin of one cell (arrows) and of the culture dish surface. (This entire process is reversible, and removal of the ethacrynic acid [or the addition of calcium] can induce the reassembly of junctions and

filopodial re-formation). Between the arrows and arrowheads in the lower part of this figure, the lateral wall of this cell is visible, depicting the entire height of the paracellular pathway, corresponding to the cell thickness. This perspective allows one to see clearly that the ZO-1 junctional protein is in fact localized along the apical most margin of the lateral wall of a given cell. Thus this image allows us to confirm that the intercellular junctions in SCEs are in fact located apically as described above. Further, as indicated by the arrowheads in the upper and lower parts of the widened intercellular space, the ZO-1 protein is still visible as punctate structures arranged in a linear disposition. Such "linearization" of the ZO-1 proteins signals the fact that the intercellular junctions have become disassembled. Also note that "linearization" occurs in TMEs exposed to ethacrynic acid or calcium-free media (data not shown). Thus, although the intercellular junctions are disassembled, the ZO-1 protein, which is located normally along the cytoplasmic aspect of the cell membrane,[13,15] remains visible inside the cell. The widened space extending between the 4 arrowheads in the lower right panel represents the width of the markedly widened paracellular pathway.

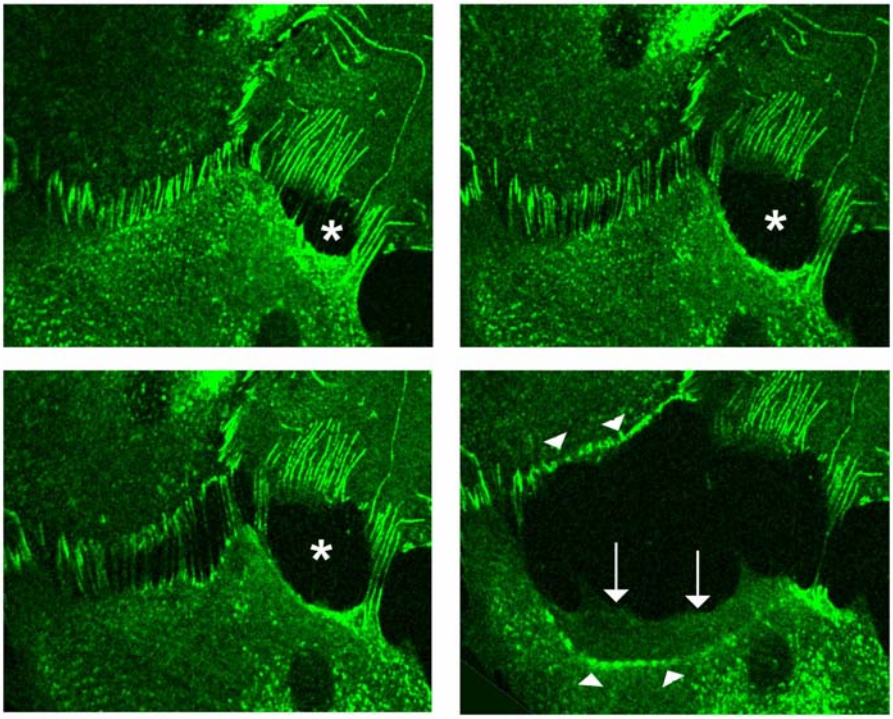

FIGURE 3

Snapshots showing the junction disassembly process in Schlemm canal endothelial cells (SCEs). SCE-junction disassembly process was induced by exposure to 0.8 mM ethacrynic acid added to the culture media, and documentation was carried out during a 40-minute time-lapse confocal microscopy session. Snapshots presenting the disassembly process were taken at 0 minutes (upper left), at 22 minutes (upper right), at 32 minutes (lower left), and at 37 minutes (lower right) (original magnification ×40).

TREATMENT WITH THE FREQUENCY-DOUBLED ND:YAG LASER

The transfected SCEs were exposed to media conditioned by lasered TMEs, which we had shown to increase permeability across SCEs.[12,17] The SCEs were then examined at 24 and 48 hours using confocal microscopy and compared to control SCEs cultured in standard media. Figure 4 (upper left) shows the control SCEs examined after 48 hours of exposure to standard medium. The filopodia are fully formed and extend across the intercellular space, and they hold adjacent cells closely together (similar findings were obtained after 24 hours of exposure to the same control media, data not shown). In contrast, SCEs exposed for 24 hours to media conditioned by lasered-TMEs (upper right panel) show less prominent filopodia, which at 48 hours have largely disappeared and assumed a linear disposition (lower left panel). The lower right panel shows at a higher magnification (63×) a portion of the intercellular junction depicted in the middle left panel. Note that the junctions have assumed a linear appearance, which we propose signals the termination of the junction disassembly process. The use of immunofluorescence methods yielded similar results as observed in experiments using nontransfected cells (data not shown).

We then examined the effect of the conditioned media on the permeability of SCE monolayers (Figure 5). There was a fourfold increase in conductivity compared to nontreated controls, induced by adding media conditioned by lasered TMEs onto SCEs, while there was a threefold conductivity increase induced by lasering SCEs directly. These physiologic data, together with the junction-disassembly morphologic experiments, support the concept that the lasering procedure induces the junction-disassembly and the corresponding major permeability increases.

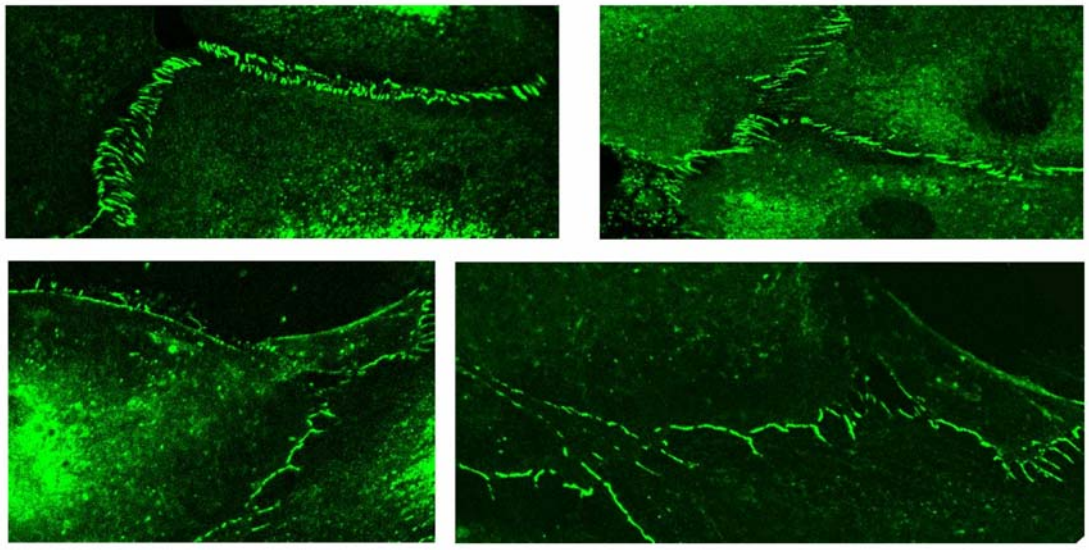

FIGURE 4

The effect of media conditioned by lasered trabecular meshwork endothelial cells (TMEs) on the Schlemm canal endothelial cell (SCE) barrier. Media conditioned by lasered TMEs were added for up to a 48-hour period onto SCE to monitor the response of the SCE barrier. The untreated control (upper left) is compared with the response at 24 hours (upper right) and 48 hours (lower left and lower right) in treated preparations (images in the upper left, upper right, and lower left panels, original magnification ×40; lower right panel, ×63 magnification).

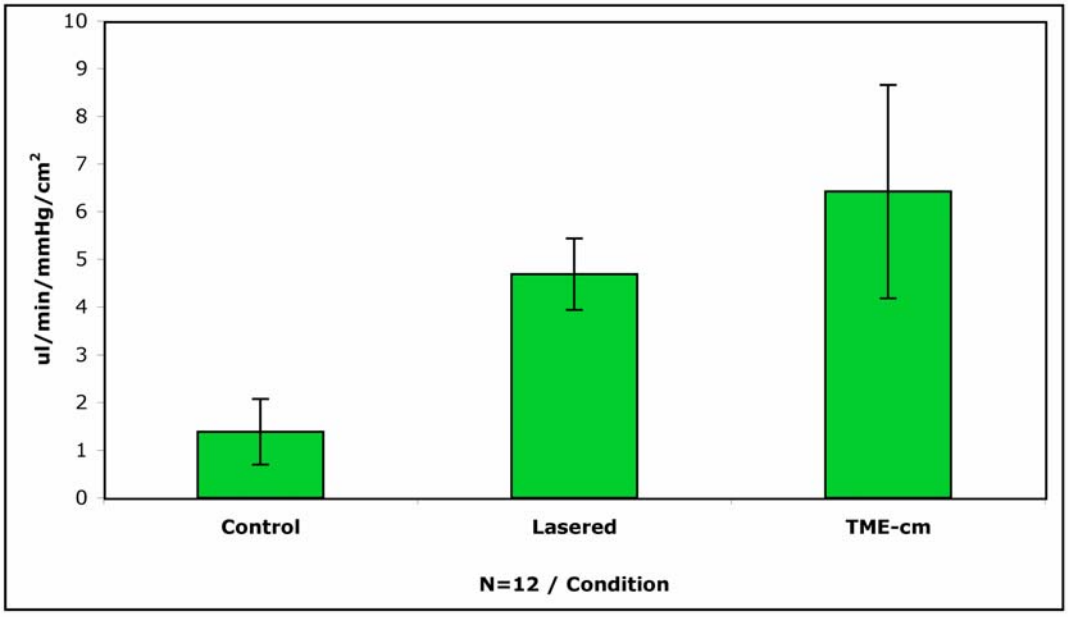

FIGURE 5

Change in conductivity in Schlemm canal endothelial cells (SCEs) undergoing direct laser irradiation using the Frequency-Doubled Nd:YAG instrument or exposed to media conditioned (cm) by lasered trabecular meshwork endothelial cells (TME-cm). The SCE-conductivity effects of the laser treatment and the addition of media conditioned by lasered TMEs are depicted. Compared to controls, laser treatment of SCEs induced a threefold increase in conductivity, and the conditioned media induced a fourfold increase.

EXPOSURE TO PROSTAGLANDIN ANALOGUES

Transfected SCEs were exposed to one of three PGAs while monitoring the intercellular junction status. Figure 6 presents snapshots from an experiment in which SCEs were exposed to latanoprost or travoprost for 12 hours and compared to untreated controls. The left panel shows the appearance of SCEs in untreated controls, where the filopodia are easily discernible at the end of incubation for 12 hours. In the middle panel, a group of cells is shown that have become elongated after treatment with latanoprost and where no filopodia are apparent, as the junctions have undergone disassembly and the ZO-1 proteins have assumed the linear disposition characteristic of disassembled junctions. In the right panel, the result after travoprost exposure is shown, which again demonstrates elongation of the cells and absence of filopodia. Similar morphologic and junction effects were observed in bimatoprost-treated cells (data not shown). These results were confirmed using immunofluorescence assays on nontransfected cells (data not shown).

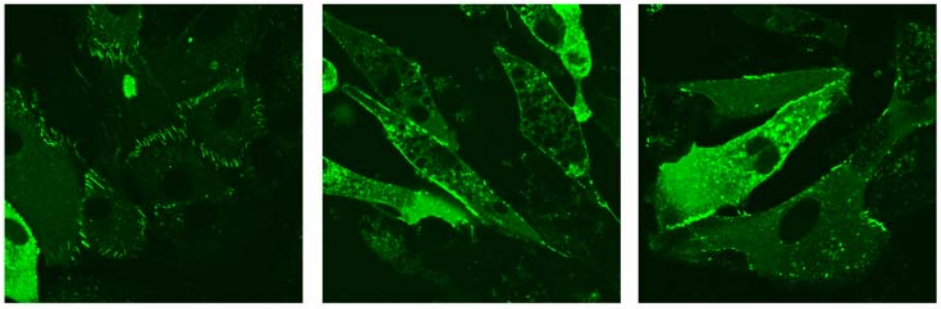

FIGURE 6

Prostaglandin analogue–induced junction disassembly in Schlemm canal endothelial cells (SCEs). The normal appearance of filopodia and the square shape of SCEs are shown in the left panel for the nontreated cells. The elongated appearance and the absence of filopodia are shown in preparations exposed to latanoprost (middle panel) and travoprost (right panel) (original magnification ×40)..

We then measured the permeability across SCE monolayers treated with PGAs for 12 hours. Figure 7 shows first the conductivity in control untreated preparations at the outset of the experiment and 12 hours later, with no discernible change in conductivity detected. However, preparations receiving bimatoprost and latanoprost showed a nearly twofold and a fourfold increase, respectively. Thus, congruous effects were measured in assays evaluating the junction-disassembly process and in those measuring conductivity changes. These data support the contention that PGAs have similar morphologic and physiologic effects as irradiation when using a frequency-doubled Nd:YAG laser employing parameters similar to those used clinically during SLT procedures.

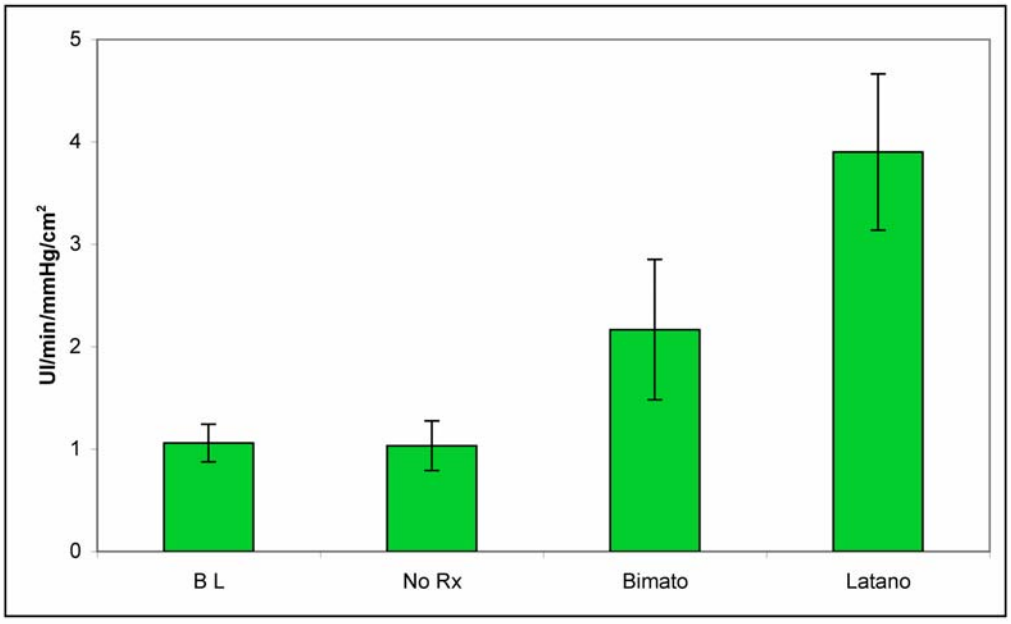

FIGURE 7

Change in conductivity of Schlemm canal endothelial cells treated with 2 prostaglandin analogue medications. The conductivity at baseline (BL) is compared with untreated controls (No Rx) and those exposed to bimatoprost (Bimato) and latanoprost (Latano) at the end of the 12-hour experimental period.

In contrast to the results with these PGA assays, the addition of brimonidine, timolol, or brinzolamide to SCEs induced no alteration of the filopodia. This could be observed using immunofluorescence for ZO-1 as shown in Figure 8 (data shown only for brimonidine). In the left panel, the appearance of SCEs in untreated controls is shown for comparison with the right panel, which shows that brimonidine induced no alterations in the appearance of filopodia in treated SCEs after exposure for 12 hours. A similar lack of morphologic effect was detectable after the addition of timolol or brinzolamide (data not shown).

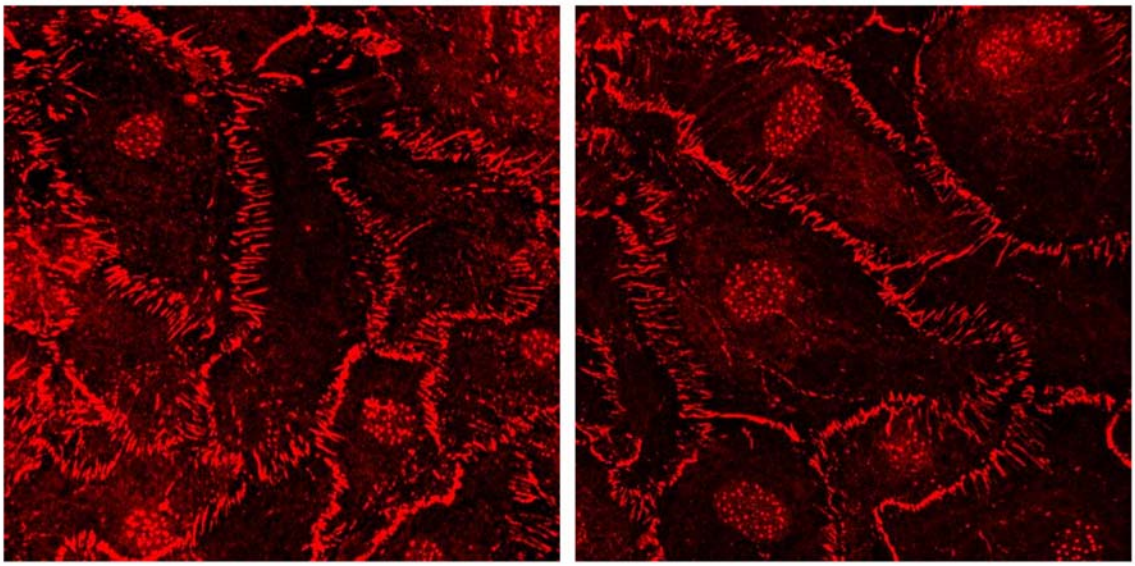

FIGURE 8

The addition of non−prostaglandin analogue medications induces no filopodial alterations in Schlemm canal endothelial cells (SCEs). SCE control (left panel) and experimental preparations (right panel) are compared (original magnification ×40). The results show no discernible differences in the filopodial status and the cell shape after treatment with brimonidine. Similar results were obtained in preparations treated with timolol and brinzolamide.

CLINICAL STUDIES

Our sample of eyes consisted of the first SLT-treated eye for each of 24 consecutive patients who had been treated previously with PGA therapy. Table 1 presents the raw IOP data for each eye in the sample under 3 conditions: during PGA therapy (PGA), off PGA therapy (BASELINE), and approximately 90 days after the SLT application (SLT). Although the IOP measured at the time of removal of PGA therapy was temporally prior to the IOP measured immediately preceding the SLT application, we view the IOP taken immediately preceding the SLT application as the "baseline" IOP, since it reflects IOP in the absence of either PGA or SLT therapy. Table 1 also presents the percentage difference between post-SLT and BASELINE IOP. The primary purpose for obtaining these clinical observations was to evaluate whether SLT therapy is as effective as PGA therapy in reducing IOP. Tables 2 through 5 provide results on IOP differences among the 3 measurement conditions just described.

In Table 2, average IOP is 15.94 mm Hg while on PGA therapy, 21.52 mm Hg at BASELINE while off PGA therapy, and 14.92 mm Hg ~90 days post-SLT. To evaluate whether these differences are statistically discernible, Table 3 presents results based on "paired differences" in IOP for an individual eye under different conditions. For example, row 3 of Table 3 reports the mean, standard deviation, and *P* value of the difference in IOP between 90 days post-SLT and while on PGA therapy by subtracting IOP while on PGA therapy from IOP 90 days post-SLT *for each eye*. Since our study design measures IOP in the same eyes under 3 different conditions, one of the major advantages is that the effects of different therapies can be evaluated using the same set of eyes rather than independent sets of eyes for each condition. Table 3 shows that these differences in IOP are statistically discernible: average IOP is 5.58 mm Hg lower while on PGA therapy than at BASELINE ($P < .001$); 6.60 mm Hg lower after the SLT procedure than at BASELINE ($P < .001$); and 1.02 mm Hg lower after the SLT procedure than while on PGA therapy ($P = .011$).

We view the -5.58 mm Hg difference in IOP while on PGA therapy compared to BASELINE as the average therapeutic effect of PGA administration on IOP. Although the average IOP is estimated to be 1.02 mm Hg lower after the SLT procedure than while on PGA therapy, and this appears to be statistically discernible, the magnitude of this difference is only approximately 1 mm Hg, indicating that the post-SLT IOP is, on average, very close to the level while on PGA therapy. We take this as evidence that SLT therapy is at least as effective as PGA therapy in reducing IOP. Table 3 also shows that IOP, on average, is 25.37% lower while on PGA therapy than at BASELINE ($P < .001$); 29.93% lower after SLT than at BASELINE ($P < .001$); and 5.33% lower after SLT than while on PGA therapy ($P = .031$).

Various published studies have observed a relationship between baseline IOP and post-SLT IOP reduction[1,3,4]: the higher the

TABLE 1. IOP IN 24 EYES FROM 24 PATIENTS TREATED WITH SLT AFTER STOPPING PGAs

SER. NO.	EYE	PGA	BASELINE	SLT	IOP Δ (SLT vs BASELINE)
1	OD	12	17	11	35%
2	OD	17	23	16	30%
3	OD	15	18	14	22%
4	OD	15	25	16	36%
5	OD	19	28	17	39%
6	OS	19	24	14	42%
7	OD	15	17	13.5	21%
8	OS	15	23	16	30%
9	OS	11	16	12	25%
10	OS	15	20	15	25%
11	OD	15	23	16	30%
12	OD	18	22	16	27%
13	OS	17	20	16	20%
14	OD	13.5	19	13	32%
15	OS	18	25	14	44%
16	OS	19	29	18	38%
17	OS	16	22	17	23%
18	OD	14	21	15	29%
19	OD	18	23	14	39%
20	OS	18	23	15.5	33%
21	OS	12	19	15	21%
22	OD	18	23	16	30%
23	OS	16.5	19	15	21%
24	OD	16.5	17.5	13	26%

IOP, intraocular pressure; PGA, prostaglandin analogue; SLT, selective laser trabeculoplasty.

TABLE 2. IOP (MM HG) FOR THE THREE CONDITIONS INDICATED

N EYES = 24	MEAN (SD)	MEDIAN (MIN, MAX)
IOP_{PGA}	15.94 (2.31)	16.25 (11, 19)
$IOP_{BASELINE}$	21.52 (3.40)	22.00 (16, 29)
IOP_{SLT}	14.92 (1.63)	15.25 (11, 18)

IOP, intraocular pressure; N, number; SD, standard deviation; Min, minimum; Max, maximum; PGA, prostaglandin analogue; SLT, selective laser trabeculoplasty.

TABLE 3. IOP DIFFERENCE (MM HG) BETWEEN CONDITIONS

N EYES = 24	A. MEAN DIFFERENCE (SD)	B. MEAN PERCENT DIFFERENCE (SD)
IOP_{PGA}-$IOP_{BASELINE}$	-5.58 (2.38); $P < .001$*	-25.37% (8.86); $P < .001$
IOP_{SLT}-$IOP_{BASELINE}$	-6.60 (2.44); $P < .001$	-29.93% (7.05); $P < .001$
IOP_{SLT}-IOP_{PGA}	-1.02 (1.81); $P = .011$	-5.33% (11.39); $P = .031$

IOP, intraocular pressure; N, number; SD, standard deviation; PGA, prostaglandin analogue; SLT, selective laser trabeculoplasty.
*All *P* values are for the paired *t* statistic and are 2-sided.

baseline IOP, the greater the reduction. To address this issue, Tables 4 and 5 present IOP results when the data is broken into 2 groups depending on whether the IOP is above or below 20 mm Hg at BASELINE. Tables 4 and 5 show that average IOP, both while on PGA therapy and 90 days post-SLT, is lower than at BASELINE for both groups of eyes, but is noticeably lower for the >20 mm Hg

group than for the ≤20 mm Hg group. In Table 5, for the ≤20 mm Hg group, IOP while on PGA therapy was 3.90 mm Hg lower than at BASELINE and post-SLT IOP was 4.50 mm Hg lower; for the >20 mm Hg group, IOP while on PGA therapy was 6.79 mm Hg lower than at BASELINE and post-SLT IOP was 8.11 mm Hg lower. Table 5 also provides percentage change results complementing what we see in the mean differences. The *P* values for Table 5 are not very meaningful since the baseline IOP grouping was arbitrary and was constructed only to provide for a rough evaluation of the baseline IOP effect on IOP reduction. What is important is that these tables do provide a basis for suggesting a relationship between baseline IOP and IOP reduction both for PGA therapy and SLT irradiation.

TABLE 4. IOP (MM HG) FOR THE THREE CONDITIONS, BY BASELINE IOP GROUP

		MEAN (SD)	MEDIAN (MIN, MAX)
≤20 mm Hg (N = 10)	IOP_{PGA}	14.35 (2.12)	15.00 (11, 17)
	$IOP_{BASELINE}$	18.25 (1.36)	18.50 (16, 20)
	IOP_{SLT}	13.75 (1.55)	13.75 (11, 16)
> 20 mm Hg (N = 14)	IOP_{PGA}	17.07 (1.73)	18.00 (14, 19)
	$IOP_{BASELINE}$	23.86 (2.25)	23.00 (21, 29)
	IOP_{SLT}	15.75 (1.12)	16.00 (14, 18)

IOP, intraocular pressure; N, number; SD, standard deviation; Min, minimum; Max, maximum; PGA, prostaglandin analogue; SLT, selective laser trabeculoplasty.

TABLE 5. IOP DIFFERENCES (MM HG) BETWEEN CONDITIONS, BY BASELINE IOP GROUP

		A. MEAN DIFFERENCE (SD)	B. MEAN PERCENT DIFFERENCE (SD)
≤20 mm Hg (N = 10)	IOP_{PGA}-$IOP_{BASELINE}$	-3.90 (1.87)	-21.38 (10.22)
		$P < .001$*	$P < .001$
	IOP_{SLT}-$IOP_{BASELINE}$	-4.50 (0.88)	-24.75 (5.08)
		$P < .001$	$P < .001$
	IOP_{SLT}-IOP_{PGA}	-0.60 (1.71)	-3.08 (12.51)
		$P = .297$	$P = .456$
> 20 mm Hg (N = 14)	IOP_{PGA}-$IOP_{BASELINE}$	-6.79 (1.97)	-28.22 (6.75)
		$P < .001$	$P < .001$
	IOP_{SLT}-$IOP_{BASELINE}$	-8.11 (2.04)	-33.64 (5.87)
		$P < .001$	$P < .001$
	IOP_{SLT}-IOP_{PGA}	-1.32 (1.88)	-6.93 (10.70)
		$P = .021$	$P = .031$

IOP, intraocular pressure; N, number; SD, standard deviation; PGA, prostaglandin analogue; SLT, selective laser trabeculoplasty.
*All *P* values are for the paired *t* statistic and are 2-sided.

To examine the relationship between baseline IOP and IOP reduction after therapy, we carried out 2 regression analyses: the first regressed PGA IOP-BASELINE IOP on BASELINE IOP, and the second regressed post-SLT IOP-BASELINE IOP on BASELINE IOP. For both regressions, we centered BASELINE IOP at its mean value of 21.52 so that the intercept in the regression could be

interpreted to indicate the average IOP reduction at the baseline sample mean. Table 6 shows the results for these 2 regression analyses. For both PGA therapy and SLT therapy there is a clear linear relationship between baseline IOP and the reduction in IOP from baseline. For PGA therapy, each 1 mm Hg increase in BASELINE IOP increases the therapeutic effect by 0.51 mm Hg ($P < .0001$); for SLT therapy, each 1 mm Hg increase in BASELINE IOP increases the therapeutic effect by 0.64 mm Hg ($P < .0001$). There was no nonlinearity detected in the effect of baseline IOP for either therapy. Table 7 presents, for both regressions, the 95% confidence limits for mean level of IOP reduction at selected values of baseline IOP. Since there is great overlap in the intervals for PGA and SLT, it can be seen that the 2 therapies appear to be essentially equivalent throughout the range of baseline IOP values considered.

TABLE 6. REGRESSION ANALYSES

N EYES = 24	A. $IOP_{PGA} - IOP_{BASELINE}$	B. $IOP_{SLT} - IOP_{BASELINE}$
Intercept	-5.58 (0.34)* $P < .0001$	-6.60 (0.23) $P < .0001$
$IOP_{BASELINE} - 21.52$	-0.51 (0.10) $P < .0001$	-0.64 (0.07) $P < .0001$
	Root MSE = 1.65, $R^2 = 0.54$	Root MSE = 1.11, $R^2 = 0.80$

IOP, intraocular pressure; MSE, mean square error; N, number; PGA, prostaglandin analogue; SLT, selective laser trabeculoplasty
*Estimate (standard error).

TABLE 7. 95% CONFIDENCE LIMITS FOR DIFFERENCES FROM BASELINE IOP (mm Hg)

BASELINE IOP	PREDICTED $IOP_{PGA} - IOP_{BASELINE}$	LOWER LIMIT	UPPER LIMIT	PREDICTED $IOP_{SLT} - IOP_{BASELINE}$	LOWER LIMIT	UPPER LIMIT
16	-2.74	-4.10	-1.39	-3.06	-3.97	-2.14
17	-3.26	-4.44	-2.08	-3.70	-4.50	-2.90
18	-3.77	-4.79	-2.75	-4.34	-5.03	-3.66
19	-4.29	-5.16	-3.41	-4.98	-5.58	-4.39
20	-4.80	-5.57	-4.03	-5.63	-6.15	-5.11
21	-5.32	-6.02	-4.61	-6.27	-6.75	-5.79
22	-5.83	-6.54	-5.12	-6.91	-7.39	-6.44
23	-6.34	-7.11	-5.58	-7.55	-8.07	-7.04
24	-6.86	-7.73	-5.99	-8.20	-8.79	-7.61
25	-7.37	-8.38	-6.36	-8.84	-9.52	-8.16
26	-7.89	-9.06	-6.72	-9.48	-10.27	-8.69
27	-8.40	-9.75	-7.06	-10.12	-11.03	-9.21
28	-8.92	-10.45	-7.39	-10.77	-11.80	-9.73
29	-9.43	-11.15	-7.71	-11.41	-12.57	-10.25

IOP, intraocular pressure; PGA, prostaglandin analogue; SLT, selective laser trabeculoplasty.

DISCUSSION

This is a translational research project that relates observations made in the eye clinic with those made in the research laboratory, and then tests the validity of the basic laboratory findings with subsequent observations made again in the clinical setting. This study has emphasized a translational goal: predicting SLT outcomes in terms of both the IOP decrease and success rate attained. We first learned in the laboratory that SLT and PGAs might share a common mechanism of action in regulating both the integrity of the intercellular junctions and the permeability of the barrier formed by SCEs. We then attempted to correlate our in vitro laboratory findings with clinical studies where PGA therapy was discontinued prior to SLT to avoid competition over a common mechanism of action. In our series of 24 patients, we demonstrated that this maneuver resulted in both a greater IOP-lowering effect and a higher success rate than

we had observed prior to this change in protocol. Moreover, it became apparent that the outcomes of SLT treatment were predictable based on the a priori determination that PGAs induced a decrease in the IOP in a prospective SLT candidate. That is, if PGAs induce a decrease in the IOP, this predicts both a positive response to the SLT procedure and the magnitude of the pressure-lowering effect that will be achieved. We found that the IOP decrease after laser irradiation was similar to the measured increase in IOP with the withdrawal of PGA therapy (Table 3). That is, the lack of a PGA-induced IOP-lowering response would be predictable of a similar lack of a pressure-lowering effect upon the subsequent performance of an SLT procedure on a given patient.

In the 24 patients reported here, there was an average IOP-lowering response with PGAs of 25.37%, while SLT irradiation produced an IOP decrease of 29.93% (see Table 3). Our clinical data also show that the IOP at baseline (ie, before SLT) predicts the magnitude of the IOP decrease. As shown in Table 7, for a patient with a baseline IOP of 22 mm Hg, PGA therapy would be expected to lower the IOP by 5.8 mm Hg, and SLT alone would be predicted to lower the IOP by 6.9 mm Hg. By comparison, in a cohort of patients studied previously where PGAs were not discontinued prior to SLT treatment, there was a ~30% failure rate and a <20% IOP lowering (our unpublished observations). These poor outcomes are similar to previously published reports on SLT therapy.[1] Thus, we now routinely discontinue PGAs prior to SLT in order to predict and to improve the response to SLT therapy.

Our laboratory research considers cellular/molecular mechanisms regulating transendothelial fluid flow across the barrier formed by SCEs. The capacity of the SCE-barrier in controlling the egress of aqueous outflow is supported simply by noting the marked widening of this barrier under the influence of a variety of agents. This widening is accompanied by measured changes in conductivity after the addition of PGAs, the application of media conditioned by TMEs that had undergone laser irradiation, or the direct lasering of SCEs. There was a measured threefold increase in conductivity after the direct application of SLT irradiation on SCEs, and a fourfold increase when SCEs were exposed to media conditioned by laser-irradiated TMEs, in support of prior reports from our laboratory.[12,17] By way of comparison, the application of PGAs at the doses described increased the SCE conductivity by a threefold average (ie, bimatoprost yielded a twofold change, and latanoprost induced a fourfold change). We concluded from these limited in vitro assessments that both the SLT procedure and the application of PGAs induce similar increases in conductivity. The measured conductivity changes were accompanied by the disassembly of intercellular junctions, which was documented to occur in lasered and PGA-treated preparations. Interestingly, the 3 topical non-PGA medications tested (timolol, brimonidine, and brinzolamide) yielded no alterations in the status of intercellular junction or conductivity (conductivity data not presented).

Our results indicate the existence of direct PGA effects on the SCE-barrier function and thus the conventional trabecular meshwork aqueous outflow pathway. These results are contrary to most prevailing tonography reports in the literature. Traditional early tonography studies supported a PGA mechanism of action through the pressure-insensitive uveoscleral pathway and matrix metalloproteinase effects.[27-30] However, recent studies provide a different viewpoint. In vitro perfusion studies, using anterior segments in which the uveoscleral pathway is removed by dissection, showed that PGAs do increase flow across the conventional trabecular meshwork aqueous outflow pathway.[7] Similarly, recent tonography data have shown that the 3 PGAs used in our studies do increase aqueous outflow through the conventional or trabecular aqueous outflow pathway, but the proportion due to uveoscleral and conventional outflow varies with the measurement technique used.[6] Our results also indicate the existence of SCE-barrier effects induced by the laser irradiation of SCEs and TMEs, likely associated with the release of factors mediated by an autocrine process in the case of SCEs, and a paracrine effect in the case of TMEs.[12,17] Regarding tonography studies, the early reports in the case of ALT found a 65% increase in facility and a 22% decrease in IOP 1 month post-ALT.[31-33] Most recently, a group of English investigators have measured tonographic outflow facility in SLT-treated patients and found a marked increase in facility in patients receiving a 360° SLT treatment (Lim KS, written communication, April 28, 2009). Thus, our in vitro findings are supported by the most recent tonography studies examining the effects of both PGAs and SLT.

In conclusion, we propose that our in vitro studies demonstrate a common mechanism of action for SLT and PGA therapy. Our small series of patients corroborates this laboratory finding by showing a similar magnitude of IOP reduction for both therapies. We hope that our laboratory findings in combination with our clinical results will encourage others to re-examine the use of PGA therapy in conjunction with SLT treatment. It is possible that combining PGA therapy with SLT leads to suboptimal outcomes, and providers may wish to consider alternative topical medications in patients who have received SLT therapy.

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PEER DISCUSSION

DR. JAMES C. TSAI: Selective laser trabeculoplasty (SLT), utilizing a frequency-doubled Nd:YAG laser, has been shown to possess equivalent intraocular pressure (IOP) effects to that of argon laser trabeculoplasty (ALT).[1] While ALT increases trabecular outflow,[2] the mechanism of action of SLT is currently not clearly elucidated. In this paper, the authors presented novel in vitro and clinical patient data to support the hypothesis that SLT and topical prostaglandin analogue (PGA) therapy share a similar mechanism of action of enhancing trabecular outflow. They performed sophisticated in vitro cell culture studies involving Schlemm's canal endothelial cells (SCEs) transfected with a plasmid construct containing tagged-genes for the zonula occludens-1 protein (ZO-1) and the green fluorescent protein (GFP). Time-lapse confocal microscopy documented similar degrees of junction-disassembly and corresponding increases in SCE-barrier permeability in cell cultures treated by addition of media conditioned by lasered trabecular meshwork endothelial cells (TMEs) and by direct topical application of three PGAs (latanoprost, bimatoprost, travoprost). In contrast, topical exposure of the transfected SCEs to non-PGA agents (brimonidine, timolol, brinzolamide) did not elicit the filopodial morphological alterations seen with SLT- or PGA-treated cultures. The authors also presented IOP data from a prospectively-designed but retrospectively-obtained clinical study in patients washed out from PGA therapy and subjected to SLT.

The methods employed in the in vitro experiments were rigorous, thorough, and innovative. The confocal laser scanning microscope techniques provided impressive real-time depictions of the junction assembly/disassembly process in SCEs. Key assumptions made by the investigators included the following: (1) their in vitro experiments simulated biological effects observed in the in vivo trabecular outflow system and (2) the topical drug applications applied to transfected SCEs and laser treated TME media simulated in vivo conditions in aqueous humor. Thus, a valid criticism exists as to whether the experimental conditions obtained with either single drop applications or laser-treated media reflect true and/or ideal treatment conditions, such as an optimal dose-response concentration of drugs.

In transitioning from the bench to the bedside, the investigators analyzed pre- and post-SLT IOP data of patients enrolled from a tertiary care practice and washed out from PGA therapy. The number of subjects excluded based on absence of IOP rise upon PGA withdrawal and/or less than 3 months of follow-up is unclear. In their discussion, the authors noted patient cases involving removal of non-PGA agents prior to SLT treatment wherein reinstitution of these medications demonstrated an additive effect. Thus, a stronger study design would have involved the post-SLT analysis of a separate cohort of patients washed out from non-PGA therapy.

Since PGA and SLT appeared to have similar in vitro effects, the authors hypothesized that there would be comparable IOP reductions following both therapies. This premise is consistent with findings from a randomized, prospective study comparing SLT with latanoprost for IOP control in patients with ocular hypertension and open angle glaucoma.[3] However, patient adherence to medical therapy is not always ideal, and the IOP rise obtained after discontinuing PGAs cannot be assumed to reflect the full IOP lowering effects of these agents, in contrast to post-SLT IOP readings.

Moreover, it is unclear how the authors accounted for IOP variables such as 24-hour IOP fluctuation and variation. Finally, the authors commented that the addition of PGA in the post-SLT period was not associated with additional reductions in IOP. This finding is counter to findings obtained in a recent retrospective clinical study wherein average IOP decrease was greater among PGA users compared to nonusers one month post-SLT.[4]

In summary, the authors should be commended for presenting data challenging the prevailing thinking that ALT, and by inference SLT, enhances trabecular flow, while PGAs primarily increase uveoscleral outflow. Based on innovative cell culture experiments, they have presented convincing data that PGAs have significant biological effects on the trabecular outflow system. Their clinical data suggests that comparable IOP effects are observed post-SLT with those prescribed PGA therapy and that limited additive effects should be anticipated. An alternative hypothesis that SLT possesses significant uveoscleral outflow effects comparable to those observed with PGAs (unpublished data, Goyal S and associates, American Academy of Ophthalmology 2008 Annual Meeting) suggest that there indeed may be individual differences at play. Nevertheless, the study results provide improved prognostic information to the clinician regarding the benefits of SLT in patients already on ocular hypotensive medications.

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DR. ALLAN J. FLACH**:** I have not only a bias, but a tremendous advantage. I work with Dr. Alvarado and I have been privileged to hear a long discussion of this topic and to see the film that he mentioned. He ought to bring the film next year and show it, because it is fantastic. This potentially extremely important paper may change the way we think about the pharmacodynamics of prostaglandin derivatives and analogs. In the past we were misled by the Goldmann equation and the assumptions that were made with tonography. When anterior ocular fluorophotometry was performed and provided direct measurements, we better understood the effects of timolol maleate and epinephrine HCl. Now Dr. Alvarado is suggesting we investigate further by studying histology and cell movements. This new idea is very interesting to me. My one question, Dr. Alvarado, would you please tell us a little bit about those two publications you mentioned that you did not have time to discuss, specifically the one by Johnson and the other by Lim.

DR. MALCOLM R. ING**:** I have no conflict of interest. Jorge, I have had the privilege of listening to at least one paper in which, I believe you associated a chemical effect in the aqueous humor with the SLT treatment. You then used that aqueous humor to actually decrease aqueous production or increase outflow, or at least decreased intraocular pressure. With respect to this interrelationship, could you amplify perhaps a little bit further on this possibility? The other issue relates to jumping from the bench to the practice or the bedside. Your results suggest that if you are treating a patient with glaucoma, then the treatment effect of selective laser trabeculoplasty (SLT) would be greater if performed after a wash out period of the travoprost (Travatan, Alcon Laboratories, Inc., Ft. Worth, Texas) or another prostaglandin analog, as it relates to the competitive effect of the two mechanisms. In view of the two available treatment modalities I would like to know if you now stop travoprost before treatment with SLT, and then resume medical treatment perhaps at a later date with a prostaglandin analog if the SLT does not sufficiently lower intraocular pressure.

DR. ROBERT L. STAMPER: No relevant conflicts. Jorge, this is very elegant and fascinating work. My question is actually pretty much along the lines of Malcolm's. Given the duration of effect of prostaglandins, and it is more than just perhaps a few days or even a couple of weeks, how long before the SLT would you recommend stopping the prostaglandins before proceeding with the treatment?

DR. PAUL L. KAUFMAN: I have no conflict of interest in relation to this presentation. Jorge, it is elegant work, as yours always is. The cellular and junctional findings are fascinating, but it is sort of the same question that Jim raised; does that behavior necessarily reflect what is going to happen in the much more complicated system of the trabecular meshwork? Permeability across endothelial cell layers is not the same as outflow resistance across this incredibly complex tissue. The second issue is the intraocular pressure response regarding how well the SLT and the prostaglandins work. With any physical manipulation, if you start out with a lower pressure, as you would in a PG-treated eye, the system can only go down so much, or can only go down to a certain level so it is going to look like a smaller effect. If you take away all of the other considerations then, of course the higher the starting pressure, the bigger the IOP drop for a given effect of say SLT. This is just inherent in the physics of the outflow system and the physics of the eye. The bigger they are, the harder they fall is the common way of saying that. I do think you have to take this into consideration when you say that you have some biological phenomena here going on that accounts for the 5mm Hg greater drop. I do not think I will comment on the effect of PG itself because I believe that it is really a different question and it is not the focus of the paper.

DR. JORGE A. ALVARADO: Thank you very much Dr. James Tsai for your thorough and sophisticated discussion. Dr. Tsai asked me to clarify whether our clinical study is retrospective or prospective in nature. Our study was not formally conceived as a prospective clinical trial, although in reality it represents a consecutive series of cases treated with SLT, and in that sense it could qualify as a prospective study. There are exclusion criteria described in the paper, including age, previous ocular surgery, and secondary glaucoma. The patients listed in Table 1 received the SLT procedure consecutively. In view of the improved success achieved during the treatment of the first ten patients or so, we adopted this laser treatment protocol as our new standard. Today, candidates for an SLT procedure are routinely asked to stop using prostaglandin analogues (PGAs) and usually return for an IOP check every two weeks. During this time, the IOP increases progressively to reach a maximum level around four weeks in most cases. This elevated pressure is taken to represent the baseline IOP value while not using PGAs. The patient is scheduled to have the SLT performed shortly thereafter, and subsequently, the PGA use is reinstituted for two to four weeks post SLT. By this time, the SLT becomes functional and the PGAs can be safely discontinued. According to our limited studies, an IOP rise after discontinuing the PGA predicts both a positive SLT outcome, and the level of the IOP lowering to be expected. In our hands today, we obtain approximately a 29-30% IOP reduction on the average. This response is far greater than what we achieved before implementing this new protocol.

I want to thank Dr. Allan J. Flach for his gracious comments, and for responding to my request, extended as I was running out of time during the presentation, to remind me to discuss the situation in patients receiving SLT for 180^0 and PGAs. Scherer (Reference No. 4 cited above by Dr. Tsai) has proposed that "PGA use made SLT more effective," which is contrary to our proposal that PGAs

and SLT do not seem to be additive to each other in lowering the IOP. Scherer made the SLT/PGA comparison at only one month after SLT. At this time, and depending on the baseline IOP, the SLT effect is only starting to become apparent, while the PGA has been fully functional all along. In fact, we routinely keep our patients on PGAs during the early post-SLT period to protect them from any IOP elevation, as it is often observed during this time. Thus, we believe that waiting three months after SLT is preferable, and withdrawing the PGA at that time may yield a different outcome compared to the situation at one-month after SLT. In our cases, we have observed that the IOP upon discontinuation of the PGAs is maintained at nearly the same level as measured when the PGAs were on board.

In addition, when only 180^0 of laser irradiation is used, the untreated trabecular meshwork (TM) in the other 180^0 remains fully available for the PGAs to have their effect in promoting outflow of aqueous. In fact, when treating for 180^0 with only 50 laser pulses, the untreated TM tissues between the laser shots also remain available for the PGAs to lower the IOP. If this is truly the case, even when treating for 360^0 and delivering the customary 100 laser pulses, the addition of PGAs should also induce an additional lowering of the IOP, albeit this might be a decrease of perhaps only 2-3 mm Hg. Following the same reasoning, one may predict that by delivering 150-200 low-power pulses instead, approximately 0.6 mJ, for 360^0 would reduce or perhaps even eliminate the additional IOP lowering induced by PGAs. That is, when the TM is truly lasered fully, and if the PGAs and SLT have a common effect, then the addition of PGAs might be expected to be superfluous. Thus, perhaps we have serendipitously stumbled on a novel way to determine empirically the number of laser shots, for example, 100 versus 150 or more, that constitute a complete laser-irradiation treatment. Determination of that maneuver was unavailable before this investigation.

Dr. Malcolm R. Ing's first question refers to our published work (reference #17 in this paper) in which we showed, as he mentioned, that the conventional aqueous outflow pathway has an elaborate cell-to-cell signaling pathway, whereby the various cell types in this pathway communicate with each other to maintain aqueous outflow homeostasis. In another paper, recently submitted, we have also pointed out that monocytes, which can be recruited to the TM by chemoattractant factors secreted by trabecular meshwork endothelial cells (TMEs), also participate in aqueous outflow homeostasis by modulating the barrier property of Schlemm's canal endothelial cells (SCEs). His second question refers to the specifics of our SLT treatment protocol, which he very well described already, and which is now described above in my reply to Dr. James Tsai's and below to Dr. Stamper's comments.

Dr. Robert Stamper wants to know how long before the SLT would we recommend stopping the prostaglandins before proceeding with treatment. Again, this issue is covered above in my reply to Dr. Tsai's comments. Bob, I generally wait four weeks so that I can obtain a measure of the PGA IOP lowering effect. This measurement helps to reassure the patient that the SLT procedure is highly likely to be effective, and to give the patient a ballpark value as to what the IOP-lowering effect of the laser procedure is likely to be. However, in general practice, one does not generally need this information, and I think it would be perfectly reasonable to treat even while the patient is still using PGAs. The disadvantage might be that the treating physician might detect only a modest IOP lowering effect, as we have done in the past. Again, another advantage, as mentioned above is that the treating physician, by stopping and restarting the PGA use, might learn whether the SLT procedure, and specifically the number of shots delivered, treated the entire TM fully.

With regards to the important issues raised by Dr. Paul K. Kaufman, we agree wholeheartedly that the *in vivo* situation is immensely more complex than that encountered in the laboratory. However, we remain much more encouraged than he does for several reasons. We tested the responses of SCEs *in vitro*, both in terms of any effects on permeability and the disassembly of intercellular junctions, to timolol, brimonidine, brinzolamide, and three commonly used PGAs. Among all of those several agents, only the PGAs, which represent the one glaucoma therapy that increases aqueous outflow, turned out to be the agents that uniformly increase the conductivity of the SCEs and the disassembly of their barrier. Additionally, after learning that media conditioned by lasered TMEs have a very similar effect, as do PGAs, we proceeded to show that clinically the IOP lowering effects of laser irradiation and the application of PGAs are remarkably similar. Thus, while translating findings in the laboratory into the clinical arena is seldom possible, in the circumstance of SLT and PGAs it may be possible to ascertain whether the effect of each approach is more or less the same, or nearly identical. In fact, we hope that others will be encouraged to learn whether the similar clinical responses to the SLT and PGA therapies merely represent a baseline related effect, as suggested by Dr. Kaufman, or reflect the existence of a common mechanism of action. Finally, Dr. Kaufman wants to know the dosage of the agents used, which actually were in the "single digit" μM levels for latanoprost (Xalatan, Pfizer Ophthalmics, New York New York) and travoprost (Travatan, Alcon Laboratories, Inc., Ft. Worth, Texas) (i.e. 3.5 and 2.4 μM respectively) and in the double-digit level for bimatoprost (Lumigan, Allergan, Inc. Irvine, California) (i.e. 20 μM). I think that these answers take care of all the questions. Thank you very much.