Monocyte Modulation of Aqueous Outflow and Recruitment to the Trabecular Meshwork Following Selective Laser Trabeculoplasty

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Objectives: To determine whether selective laser trabeculoplasty (SLT) induces monocyte recruitment to the trabecular meshwork (TM) in human and monkey eyes and whether monocytes increase both aqueous outflow in vivo and the conductivity of human Schlemm canal endothelial cells (SCEs) in vitro.

Methods: Monocyte recruitment was examined morphometrically in control human and monkey eyes and compared with that following SLT applied 1 to 3 days earlier. Outflow facility was measured for up to 4 days after the intracameral infusion of autologous macrophages in rabbits. Schlemm canal endothelial cell conductivity was measured using flow meters after exposing cultured SCEs to monocytes and monocyte-secreted factors for 24 hours.

Results: Our estimates show that the TM in the human eye normally had an average of 15,003 monocytes, while in the monkey eye there were 3,181 monocytes, and this number increased 4- to 5-fold following SLT. The intracameral infusion of autologous macrophages in rabbits increased outflow facility 2-fold in a rapid and sustained manner. Human monocytes and monocyte-secreted factors increased SCE conductivity 2-fold in vitro.

Conclusions: The number of monocytes/macrophages in the TM increases substantially after SLT and monocytes augment both outflow facility and SCE conductivity.

Clinical Relevance: These findings indicate that the innate immune system in general and monocytes in particular play an important role in aqueous outflow homeostasis. The recruitment of monocytes in increased numbers after SLT likely plays a role in lowering the intracocular pressure after this procedure. The intracameral introduction of autologous monocytes harvested from a vein could have therapeutic potential as a cell-based individualized treatment of glaucoma.


The presence of numerous monocytes/macrophages in the anterior chamber and in the trabecular meshwork (TM) during phacolytic glaucoma was once believed to be responsible for the intraocular pressure (IOP) elevation in this condition. However, recent studies have shed new light in support of a different role for monocytes where they may actually restore and maintain aqueous outflow homeostasis. In multiple animal models, the introduction of particulate matter into the anterior chamber led to an early IOP rise that was relieved spontaneously in association with the recruitment of monocytes/macrophages into the outflow pathway. The role of monocytes as phagocytes became apparent during the examination of the TM in a patient with pigmentary glaucoma who had received argon laser trabeculoplasty. These mononuclear phagocytes were observed ingesting melanin granules and acquiring the phenotype of pigment-laden macrophages, which then transited from the eye by passing into the lumen of the Schlemm canal to return to the systemic venous circulation. Based on these observations, we hypothesized that monocytes circulate through the TM under normal conditions and that selective laser trabeculoplasty (SLT), by inducing the release of chemokines, such as monocyte chemoattractant protein 1 and interleukin 8 (IL-8) by TM endothelial cells (TMEs), promotes the recruitment of monocytes from the circulation of the nearby iris and ciliary body tissues.

Selective laser irradiation has been in use for several years to lower the IOP, following its introduction by Latina and colleagues, as a mode of therapy for open-angle glaucoma. Its possible use as a therapeutic modality in ophthalmology was first reported by Roider and colleagues. Selective laser trabeculoplasty exerts its effects on tissues based on the principle of photothermolysis where melanin is the targeted chromophore. However, the mechanisms by which SLT...
important cellular barrier controlling the egress of aqueous.14-16 Regarding the systemic circulation, it is already known that cytokines secreted by monocytes, such as tumor necrosis factor α and IL-6, can induce the disassembly of endothelial intercellular junctions and increase permeability in the cerebral microvasculature and in umbilical veins.17-19 Based on these known monocyte-endothelial interactions in the systemic circulation, we also hypothesized that monocytes, when added directly to SCEs, can increase the permeability of the cellular barrier formed by this class of endothelial cells and thus should enhance outflow facility when infused intracameraly.

In this article, we report our findings from studies carried out to determine the number of monocytes in the TM in humans and in monkeys at baseline and their recruitment after the application of SLT. We also present our results from experiments conducted to assess the effects of monocytes on the aqueous outflow in rabbits in vitro and on the permeability of cultured human SCEs in vitro.

SELECTIVE LASER TRABECULOPLASTY

Seven eyes of 7 patients scheduled to undergo enucleation to remove a malignant choroidal melanoma either underwent SLT (n=3) or remained as untreated controls (n=4) (approved by the institutional review board of Willis Eye Institute, IRB 361). One of us (L.J.K.) performed the SLT procedure 2 to 3 days prior to a scheduled enucleation using a Q-switched, frequency-doubled Nd:YAG laser set to deliver 30 to 50 applications in 2 adjacent quadrants using standard parameters (ie, approximately 0.8 mJ in power, approximately 3 nanoseconds in duration, and 400 µm in diameter). The anterior segments of the 7 eyes were removed and were examined to determine the monocyte prevalence morphometrically in laser-treated and nonlasered control specimens.

Three monkeys were used to compare the monocyte prevalence with those obtained for the human eyes. One of these monkeys, after undergoing general anesthesia, received an SLT procedure in 1 eye consisting of 80 to 100 laser shots distributed over 360° of the TM using similar parameters as those used for the human eyes. A modified Goldman lens, which can be used in the monkey eye, was used to aim the laser beam onto the mid-TM tissues. The other 2 monkeys were nonlasered and served as controls. The 3 monkeys were killed 24 hours later and monocyte counts in the TM were carried out. For monocyte transit studies, 2 monkeys received an SLT application followed 30 minutes later by the intracameral injection of 20 µL of the AdCMV-β-galactosidase transduction construct (corresponding to 1.5 × 10⁹ plaque-forming units).20-22 While 1 nonlasered monkey received only the transduction construct and served as a control. Twenty-four hours later, the 3 monkeys were killed and the location of the labeled monocytes was traced inside the eye and other organs. The purpose of using the transduction construct was to trace, using an enzyme marker that yields a colored product, the path of the recruited monocytes as they transit through the TM into the canal of Schlemm and then to the various organs of the body. A total of 6 monkeys were used in our experiments. The University of California, San Francisco (UCSF), Institutional Animal Care and Use Committee approved the monkey protocols.

DETERMINATION OF THE RECRUITMENT OF MONOCYTES TO THE HUMAN AND MONKEY TM

The human anterior segments were fixed in paraformaldehyde, 1%, and cut meridionally into 1 block/quadrant, yielding 4 blocks per eye, with each block measuring 1.5 mm in width. Each dissected block included the entire aqueous outflow pathway, as well as some peripheral cornea and adjacent ciliary body and iris tissues. The tissue blocks were embedded in glycol methacylate and were cut into step-serial sections, each measuring 2 µm in thickness and separated from each other by a step measuring 25 µm, yielding 60 step-serial sections per block/quadrant and 240 step-sections per eye. Each section was exposed to anti-CD68 antibody (Zymed, San Francisco) for the purpose of counting CD68⁺ cells on a Zeiss ultraphotomicroscope (Zeiss, Thornwood, New York) since CD68 is a marker specific for monocytes. The monkey specimens were fixed in paraformaldehyde, 1%/glutaraldehyde, 2%, solution and embedded in Araldite adhesive (Huntsman Advanced Materials, Salt Lake City, Utah). The tissue blocks from the monkeys were also cut into sections of 2 µm in thickness, spaced at 25-µm intervals, with 60 step-serial sections per block, and they were stained with Richardson methylene blue. Using a Zeiss ultraphotomicroscope, each of the 60 sections per block was inspected and the mononuclear cells were counted as representing monocytes. For the human and monkey specimens, the mean monocyte count and the standard deviation per 60 samples for each of the examined blocks was determined. For the studies on the transit of monocytes through the monkey eye, the monocytes recruited to the anterior chamber after SLT and labeled in vivo by the nuclear-localizing β-galactosidase transduction construct were stained with the β-galactosidase substrate X-gal (Invitrogen, Carlsbad, California) and/or anti-CD68 antibody.

INTRACAMERAL INFUSION OF MACROPHAGES AND MEASUREMENT OF OUTFLOW FACILITY IN RABBITS

Autologous macrophages were harvested from the peritoneal cavity of rabbits that had received an intraperitoneal injection of 50-µL sterile light mineral oil 4 to 6 days earlier, using a similar technique as reported previously.23 The macrophages were recovered by peritoneal lavage with lactated Ringer solution and they were resuspended in AIM-V medium (GIBCO, Gaithersburg, Maryland) at a concentration of approximately 100 000 macrophages in a 30-µL aliquot. The anterior chamber of each rabbit, after appropriate sedation and anesthesia, was entered using a 27-gauge angiocatheter connected to an apparatus consisting of a Harvard perfusion pump, a reservoir set at a given driving pressure, and a pressure gauge that measured the IOP.24 Approximately 100 000 macrophages in 30-µL medium were infused at a flow rate of 5 µL/min (a perfusion rate that was chosen empirically to maintain a steady baseline IOP) into the anterior chamber of rabbit experimental eyes. The other eye of the same animal served as a control and received either an infusion of 30 µL of AIM-V medium or dead monocytes that were killed by fixation with 100% ethanol followed by a thorough wash in AIM-V medium. Otherwise, the components of the infusate, the infusion rates, and the driving pres-
Monocytes were purified from peripheral blood mononuclear cell preparations obtained from healthy volunteers (approved by the UCSF institutional review board, IRB H7023-22712-06) using the BD Aria cell sorter (BD Biosciences, Franklin Lakes, New Jersey) by sorting cells stained with anti-CD14 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting cell population was characterized phenotypically using flow cytometry to determine that only preparations that had greater than 99% CD14+ monocytes were used for our experiments. The harvested monocytes were added to human SCEs maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 2mM l-glutamine, 50 µg/mL of gentamicin, and 2.5 µg/mL of amphotericin B (all from Mediatech, Manassas, Virginia) and grown over Millipore filter supports (Millipore, Billerica, Massachusetts). Other SCE monolayers were exposed to conditioned media obtained by culturing the monocytes in standard media for 24 hours. The conductivity of SCE monolayers was measured using flow meters and a computer-driven perfusion apparatus previously described.24

RESULTS

SLT INDUCES THE RECRUITMENT OF MONOCYTES TO THE HUMAN TM

The number of monocytes in the human TM in the baseline state and following SLT application was determined in anterior segments dissected from 7 enucleated human eyes. Mononuclear cells were seen strictly localized to the aqueous channels along the intertrabecular spaces, as shown in a histological section of the lasered TM (Figure 1). We did not observe any monocytes within the collagenous connective tissue spaces within the uveoscleral cords, or the corneoscleral sheets, nor did we observe other leukocytes, including lymphocytes, within aqueous channels in the TM sections.

The results from the monocyte count studies in 3 lasered eyes and 4 control eyes based on the number of CD68+ cells are shown in Table 1. For the control eyes, we sampled 4 equidistant regions per eye (ie, 90° apart from each other), each region measuring 1.5 mm in width (for a total of 6 mm, which amounts to one-sixth of the entire TM circumference, which measures about 36 mm in circumference). For the lasered eyes, only 1 such 1.5-mm region was taken from 1 of the 2 quadrants that had received the laser irradiation. The monocyte counts of the lasered and nonlasered control eyes were compared as shown in Table 1. Control eyes had a prevalence of 15 003 monocytes in the entire TM at a given time and this prevalence increased nearly 5-fold to 74 529 in the lasered human TM. In conclusion, these human studies provide quantitative evidence that monocytes are present in the TM of normal individuals and that this number can increase 5-fold on lasering the TM of patients who do not have glaucoma.

Table 1. MC Results for 3 Lasered and 4 Control Human Eyes

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Eyes × No. of Regions a Examined</th>
<th>Mean (SD)</th>
<th>No. of Monocytes in the Entire TM c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4 × 4 = 16</td>
<td>52.096 (14.2)</td>
<td>15 002.67</td>
</tr>
<tr>
<td>Lasered</td>
<td>3 × 3 = 9</td>
<td>258.800 (22.4)</td>
<td>74 529.99</td>
</tr>
</tbody>
</table>

Abbreviations: MC, monocyte count; TM, trabecular meshwork.

a Region refers to the number of different locations surveyed in each eye. Four regions indicates that 4 separate locations 90° apart from each other were examined by counting the MC in 60 samples or sections cut within a given region, with 1 region taken per quadrant.

b The t test for lasered vs control eyes, based on results from a repeated-measures model, assuming a 1-factor variance components model for the random individual eye effect: standard error = 0.28, t = 8.10; P value < .001.

c Calculated assuming a corneal diameter of 11 mm (or 11 000 µm), so that the TM circumference = (π × 11 000 µm). The total number of monocytes in the entire TM was calculated as (π × 11 000 µm × mean MC per 60 samples) × 120 µm. The quantity 120 µm represents the width of the segment of TM tissue covered by 60 samples taken from a region, each sample measuring 2 µm in thickness.
SLT INDUCES THE RECRUITMENT OF MONOCYTES TO THE MONKEY TM

The monocyte prevalence studies carried out in human eyes were repeated in monkeys for comparison purposes. Selective laser trabeculoplasty was performed for 360° on the TM in 1 eye of 1 monkey, and 1 eye from each of 2 age-matched monkeys were used as untreated controls. Only one 1.5-mm TM region was examined from each of the 3 eyes. Monocytes could be seen in the intertrabecular spaces in the monkey TM (Figure 2). The results of the monocyte count studies carried out on those monkeys are presented in Table 2. The mean monocyte count in the nonlasered TM amounted to 3181 monocytes/eye, while in the TM of the lasered eye there were 13 195 monocytes/eye (Table 2). Although the actual number of monocytes in the monkey eye is much smaller than that in human eyes, the proportion of monocytes recruited was similar, with the number of monocytes being 4-fold greater in the lasered animal compared with controls.

Next, we carried out additional experiments to trace the path taken by monocytes by labeling them in vivo using the adenoviral transduction construct AdCMV-β-galactosidase. Two lasered monkeys and 1 nonlased monkey received an intracameral injection of a β-galactosidase transduction construct. The location of the labeled monocytes was traced inside the eye 24 hours later. Monocytes, labeled by the transduction construct, could easily be traced being recruited to the TM from the blood vessels in the neighboring uveal circulation in the iris and ciliary body in the SLT-treated eyes (Figure 3A). Similar results were obtained for the eye of the nonlased monkey injected with the transduction construct except that the density of monocytes was much less. In Figure 3B and C, monocytes are shown within the aqueous channels of 2 different sections of the TM, with the nucleus stained blue by the transduction construct and the rest of the cytoplasm stained reddish-brown by the binding of the anti-CD68 antibody. Such labeled cells could also be observed inside the lumen of the Schlemm canal, in the deep and superficial episcleral venous plexus, and in immune organs, including the spleen, thymus, and liver (data not shown). Based on these results, we conclude that monocytes enter the TM from the circulation of the iris and the ciliary body and then return to the systemic circulation by transiting through the TM with aqueous humor and entering the canal of Schlemm and the episcleral venous system.

MONOCYTES/MACROPHAGES INCREASE AQUEOUS OUTFLOW IN VIVO

Two separate sets of experiments were carried out in Dutch pigmented rabbits in a stepwise fashion. In the first set of experiments, we tested the effect of autologous macrophages on aqueous outflow in rabbits during the first 2 hours after the infusion of macrophages into the anterior chamber. One of the eyes of a rabbit received an intracameral injection containing living macrophages, while the other eye was used as a control and received an injection of killed macrophages. The results of this experiment are presented in Figure 4A. Outflow facility measured initially around 0.2 µL/min/mm Hg in both the experimental and control eyes prior to infusion, respectively, of the experimental and control solutions. However, the facility increased rapidly, reaching more than 0.5 µL/min/mm Hg at 1 hour in the eye receiving the live macrophages, decreasing only slightly to measure more than 0.4 µL/min/mm Hg after 2 hours. In the eye receiving killed macrophages, outflow facility remained at baseline levels at all the times tested. The experiment was repeated in 1 more rabbit with similar findings.

Table 2. MC Results for 1 Lasered and 2 Control Monkey Eyes

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Eyes × No. of Regionsa Examined</th>
<th>Mean (SD)</th>
<th>No. of Monocytes in the Entire TMb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2 × 1 = 2</td>
<td>13.5 (2.12132)</td>
<td>3180.86</td>
</tr>
<tr>
<td>Lasered</td>
<td>1 × 1 = 1</td>
<td>56.0 (NA)</td>
<td>13 194.68</td>
</tr>
</tbody>
</table>

Abbreviations: MC, monocyte count; NA, not applicable; TM, trabecular meshwork.
aRegion refers to the number of different locations surveyed in each eye.
bCalculated assuming a corneal diameter of 9 mm (or 9000 µm), so that the TM circumference = (π × 9000 µm). The total number of monocytes in the entire TM was calculated as (π × 9000 µm × mean MC per 60 samples ÷ 120 µm). The quantity 120 µm represents the width of the segment of TM tissue covered by 60 samples taken from a region, each sample measuring 2 µm in thickness.
In the second set of these experiments, we examined the effects of the infusion of autologous macrophages on outflow facility at the outset and 4 days after the infusion. One of the eyes of 6 experimental rabbits received live autologous macrophages, and the other eye in each of these 6 rabbits was used as a control and received a sham injection containing AIM-V medium alone. The results of this experiment are presented in Figure 4B. The baseline facility again measured around 0.2 µL/min/mm Hg in all 12 eyes (6 controls and 6 experimental eyes). After 4 days, the facility had at least doubled in the 6 rabbit eyes that received live macrophages; however, in control eyes, the facility remained at the baseline values. Therefore, we conclude that the infusion of autologous macrophages results in a marked and rapid lowering of the IOP that is sustained for at least 4 days.

**MONOCYTES INCREASE SCE PERMEABILITY IN VITRO**

In these experiments, we tested the effects of monocytes isolated from peripheral veins of human blood donors on the permeability of SCEs, a cell type that was selected for these experiments because they are generally recognized as a major source of the resistance normally presented to aqueous outflow. Monocytes or monocyte-conditioned media were added separately to confluent monolayers of SCEs grown over porous filter supports. The monocytes and their conditioned media were maintained in contact with the SCEs for 24 hours, after which the conductivity of the monolayers was measured. Both the monocyte-conditioned media when added alone (ie, without any cells) or monocytes when added directly to SCEs increased SCE permeability compared with controls.

**COMMENT**

We demonstrated that monocytes normally circulate through the conventional aqueous outflow pathway in significant numbers and that after SLT monocytes are recruited to the conventional aqueous outflow pathway in increased numbers. These mononuclear cells can increase aqueous outflow rapidly, dramatically, and for a prolonged period after their intracameral introduction. One caveat of our in vivo study is that it is possible that those macrophages have likely ingested mineral oil and could have been slightly altered. The observed in vivo effects may be related at the cellular level to a demonstrated increased permeability across hu-
man SCEs measured when these cells were exposed to monocytes and monocyte-derived factors in vitro. Considering the data presented herein together with our previously published studies\(^5,6\) provides a cohesive picture for the role of monocytes in the regulation of aqueous outflow. In addition, our findings point to the possible involvement of monocytes in mediating the IOP-lowering effect of SLT. According to this picture, monocytes are present in significant numbers in the outflow pathway, and as the principal cellular components of the innate immune system, these cells may become engaged in repair and surveillance activities in the TM. The repair activities include clearing out particulate matter that may obstruct the normal egress of aqueous humor.\(^5,6\) Surveillance activities may result in the release of molecular factors under normal conditions and following laser irradiation when using SLT. These factors may function to open the barrier formed by SCEs resulting in the lowering of the IOP to maintain aqueous outflow homeostasis. Our findings are supported by the observations of Melamed and colleagues,\(^26\) who demonstrated that a biological process is involved in the mechanism of action of argon laser trabeculoplasty.

To our knowledge, this participation of mononuclear phagocytes in the regulation of aqueous outflow and in the mechanism of action of laser trabeculoplasty has not been previously considered. This is probably linked to a long-standing belief that inflammation is generally associated with a detrimental aftermath, which, in turn, is related to the notion that monocytes/macrophages are only phagocytic cells. But in reality these cells also have a significant capability for the secretion of vasoactive cytokines to promote transendothelial fluid flow. In this regard, we propose that monocytes that circulate through the TM are engaged in cell-to-cell interactions with TMEs and SCEs, thus forming a cell module that functions in an autocrine and paracrine manner to maintain aqueous outflow as proposed in Figure 6.

According to the model presented in Figure 6, TMEs release chemokines, such as monocyte chemoattractant protein 1, CXCL6, and IL-8\(^7,25\) (A.S.S., S.T., and J.A.A., unpublished data, 2009), that recruit monocytes to the TM, which then release cytokines, such as tumor necrosis factor \(\alpha\), that could in turn modulate the functioning of TMEs or the permeability of the SCE barrier.\(^7,25\) The mononuclear

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**Figure 6.** A model of autocrine and paracrine interactions in the conventional aqueous outflow pathway. This theoretical model depicts the proposed autocrine and paracrine interactions between trabecular meshwork endothelial cells (TMEs), Schlemm canal endothelial cells (SCEs), and monocytes that are involved in the regulation of the outflow of the aqueous humor. Solid arrows indicate interactions for which there is a significant amount of supporting evidence, whereas broken arrows represent presumed interactions. IL indicates interleukin; SLT, selective laser trabeculoplasty; TNF-\(\alpha\), tumor necrosis factor \(\alpha\).
phagocytes can also clear out from the TM and the eye particulate matter that might obstruct aqueous outflow. In addition, cytokines released by TMEs, such as IL-1 and IL-6, can directly act on the SCe barrier. Moreover, cytokines released by TMEs and SCeS could function in an autocrine manner to regulate the functions of the respective cells. Selective laser trabeculoplasty could lead to the lowering of the IOP by augmenting the interactions among the 3 cell types described. We plan to conduct investigations into the cellular and molecular mechanisms by which monocytes participate in the proposed autocrine/paracrine network to maintain the normal rate of aqueous humor outflow in health and disease. Since there are different monocyte subsets in the circulation (the major subsets being CD14low/CD16+ and CD14int/CD16−) that could yield different macrophage populations, it will be interesting to identify which particular subtype of monocytes/macrophages is involved in the IOP-lowering effect of these cells.

Our findings further suggest that mononuclear phagocytes could potentially be useful for the future treatment of glaucoma. This approach could very well result in a prolonged pressure lowering due to the persistent monocyte-mediated repair and surveillance activities and their role in the maintenance of the aqueous outflow pathway in a normal/healthy state. Thus, monocytes harvested from a vein of an individual patient, when purified and reintroduced into the anterior chamber of that same patient, could work to promote aqueous outflow and to lower the IOP elevation characteristic of some types of glaucoma. Moreover, the determination of the nature and identity of the substances in the monocyte-conditioned media that increase SCe permeability could lead to the development of new therapeutic modalities for glaucoma.

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