The Influence of Genetic Background on Conventional Outflow Facility in Mice

Alexandra Boussommier-Calleja and Darryl R. Overby

Department of Bioengineering, Imperial College London, London, United Kingdom

Correspondence: Darryl R. Overby, Department of Bioengineering, Imperial College London, London SW7 2AZ, UK; d.overby@imperial.ac.uk.

Submitted: August 9, 2013

Accepted: October 29, 2013

Citation: Boussommier-Calleja A, Overby DR. The influence of genetic background on conventional outflow facility in mice. *Invest Ophthalmol Vis Sci*. 2013;54:8251–8258. DOI: 10.1167/iov.13-13025

PURPOSE. Intraocular pressure (IOP) varies between genetically distinct strains of mice. The purpose was to test the hypothesis that strain-dependent differences in IOP are attributable to differences in conventional outflow facility (C).

METHODS. The IOP was measured by rebound tonometry in conscious or anesthetized BALB/cJ, C57BL/6J, and CBA/J mice ($N = 6$ –10 per strain). Conventional outflow facility was measured by ex vivo perfusion of enucleated eyes ($N = 9$ –10 per strain).

RESULTS. Conscious IOP varied between strains, being highest in CBA/J (14.5 ± 0.9 mm Hg, mean $\pm$ SD), intermediate in C57BL/6J (12.3 ± 1.0 mm Hg), and lowest in BALB/cJ (10.6 ± 1.8 mm Hg) mice. Anesthesia reduced IOP and eliminated any detectable differences between strains. Conventional outflow facility also varied between strains, but, in contrast to IOP, C was lowest in CBA/J (0.0113 ± 0.0031 $\mu\text{L}/\text{min}/\text{mm Hg}$) and highest in BALB/cJ (0.0164 ± 0.0059 $\mu\text{L}/\text{min}/\text{mm Hg}$). Like IOP, C was intermediate in C57BL/6J (0.0147 ± 0.0029 $\mu\text{L}/\text{min}/\text{mm Hg}$). There was a strong correlation between conscious IOP and outflow resistance ($1/C$) from individual eyes across all three strains, revealing that 70% of the variation in IOP was attributable to variation in outflow resistance.

CONCLUSIONS. Differences in IOP among three genetically distinct murine strains are attributable largely to differences in conventional outflow facility. These results motivate further studies using mice to identify the morphologic and genetic factors that underlie IOP regulation within the conventional outflow pathway.

Keywords: outflow facility, trabecular meshwork, mouse model, intraocular pressure

The facility of aqueous humor drainage through the conventional or trabecular outflow pathway is a major determinant of IOP, and decreased conventional outflow facility (C) is the underlying cause of ocular hypertension associated with primary open-angle glaucoma.¹ Therefore, an improved understanding of aqueous outflow physiology may identify targetable mechanisms that may lower IOP more successfully for glaucoma therapy.

Recently, there has been growing interest in mouse models of aqueous humor outflow. Despite having a thinner and less elaborate trabecular meshwork, the mouse conventional outflow pathway is similar to that of humans by having a lamellated trabecular meshwork and a continuous Schlemm's canal,² and mice exhibit segmental outflow in the trabecular meshwork.³ The C in mice responds to compounds that affect outflow facility in humans, including TGF- β ,^{4,5} sphingosine 1-phosphate,^{6,7} latanoprost,^{8–12} steroids,^{13,14} and prostaglandin EP₄ receptor agonists.^{7,15} Like humans, mice do not appear to exhibit “washout,”¹⁶ which is the characteristic increase in C that occurs during prolonged ocular perfusion^{17,18} in most nonhuman species including monkeys.^{19,20} On the other hand, some studies^{10,21,22} (but not all⁸) have suggested that the majority of aqueous humor outflow in mice passes through the unconventional or uveoscleral outflow pathway, unlike humans. Nevertheless, in light of the anatomic and functional similarities between mice and humans in regard to conventional aqueous humor outflow, there is growing interest in

using mice as animal models to investigate the physiology of outflow regulation within the trabecular meshwork.

In mice, IOP depends on genetic background and spans nearly a 2-fold range between strains (e.g., 11 vs. 19 mm Hg between BALB/cJ versus CBA/CaJ^{23,24}). Because phenotypic differences often are linked to genomic polymorphisms,²⁵ there is an opportunity to exploit the natural phenotypic diversity of IOP to gain insight into the genetic basis of IOP regulation. According to Goldmann's equation, differences in IOP could be attributed to differences in any of four parameters describing aqueous humor dynamics: aqueous humor production rate (F_m), episcleral venous pressure (EVP), C , and unconventional outflow rate (F_u). While several studies have measured some or all of these parameters for a particular strain,^{8,10,22} few studies have measured parameters across different strains,²⁶ and post hoc comparison between studies is complicated by technical differences or environmental conditions. For example, C measured in a single inbred strain (BALB/cJ) varies 3-fold among different reports^{8,9} (0.0055 vs. 0.0180 $\mu\text{L}/\text{min}/\text{mm Hg}$). Thus, there is a need for a study that directly compares aqueous humor dynamics between different strains of mice.

The aim of this project was to test the hypothesis that differences in IOP between different strains of mice are attributable to differences in C . To test this hypothesis, we measured IOP in BALB/cJ, C57BL/6J, and CBA/J mice, three inbred strains selected based on previous studies^{23,24} as exhibiting low, medium, and high IOP. We then measured C ex vivo in enucleated eyes from the same cohort of animals, and

TABLE 1. Parameters of the Tonometer Calibration as Described by Equation 1

Strain	α	β , mm Hg	R^2
BALB/cJ, $N = 2$	1.0	-0.9	0.97
C57BL/6J, $N = 2$	0.9	0.7	0.98
CBA/J, $N = 2$	1.1	-0.1	0.95
All strains, $N = 6$	1.0	-0.1	0.96

examined the relationship between C and IOP as predicted by Goldmann's equation.

METHODS

All experiments were done in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research under UK Home Office Project License 70/7306.

Animal Husbandry

This study used three inbred strains of mice chosen as strains typically exhibiting low, medium, and high IOP^{23,24}: BALB/cJ ($N = 13$ mice), C57BL/6J ($N = 13$), and CBA/J ($N = 15$), respectively. Preliminary studies used CBA/CaJ ($N = 10$), but this strain did not exhibit as high an IOP as expected based on a prior report.²³ Therefore, we replaced CBA/CaJ with CBA/J. All mice were female and were purchased from Jackson Laboratories (Bar Harbor, ME) at 6 weeks of age. Mice were housed in individually ventilated cages, fed ad libitum, and maintained at 21°C with a 12-hour light (6 AM to 6 PM) and 12-hour dark cycle. The IOP measurements were attempted on 30 mice (10 per strain) aged between 3 and 4 months, while facility measurements were attempted on 30 mice aged between 4 and 7 months. Between IOP and facility measurements, there was an outbreak of pinworm in the room where our mice were housed. There was no evidence as to whether our mice had contracted the parasite, and fenbendazole prophylaxis was refused to avoid potential effects on facility.

IOP Measurements

The IOP was measured noninvasively using a commercially available rebound tonometer (TonoLab; ICare, Helsinki, Finland) in conscious and anesthetized mice. This tonometer measures IOP as a function of the deceleration of a lightweight probe propelled against the central cornea.²⁴ All IOP measurements were performed on the right eye between 12 PM and 4 PM to minimize the influence of diurnal variations.²⁷ The tonometer was held by hand for consecutive rebound measurements used to calculate a single IOP value, following manufacturer's instructions, and tonometer readings were repeated if there was any sign of error or large variability between consecutive rebound measurements. More specifically, readings were repeated if the "dash" indicator on the tonometer display reported medium or high variability, or if IOP values were artificially elevated (typically >20 mm Hg) due to the mouse becoming restless or stressed during the measurement.

The tonometer was calibrated in one eye of two mice from each strain to validate the tonometer readings and to account for potential interstrain differences in corneal biomechanics that may affect the rebound measurement. Calibration was performed on cadaveric eyes maintained in situ that were pressurized between 6 and 30 mm Hg via a cannula connected to an adjustable height reservoir, with the calibrated IOP determined based on the height difference between the limbus

FIGURE 1. Calibration data from the rebound tonometer using cadaveric eyes in situ (two eyes per strain). Each eye was cannulated and manometric IOP_m was controlled by a fluid reservoir, while tonometric IOP_t was measured using the "manually-calculated" method (see Methods). The calibration relationship was not different between strains, and, therefore, data from all strains were lumped together to obtain the linear regression shown in the Figure. All tonometric IOP data in this study were corrected according to the inverse of this linear regression (Equation 2). Each data point refers to a single manually-calculated IOP measurement from a single eye.

to the reservoir meniscus. The eyelids were removed and the cannula was inserted into the anterior chamber near the limbus so as to minimize interference with the tonometer probe that rebounds against the central cornea. At each of 10 pressure levels, the eye was hydrated with a drop of PBS and left to equilibrate for at least 3 minutes. The drop of PBS was removed with tissue paper before rebound measurements. There was no evidence of corneal damage after repeated IOP measurements.

During the calibration, tonometric IOP was measured using two methods. First, following manufacturer's instructions, a single IOP value was read from the tonometer display following six consecutive rebound measurements. We refer to this as the "tonometer-reported IOP." However, the manufacturer provides little information on the algorithm used to calculate the tonometer-reported IOP, and our separate calibration studies suggested that the tonometer-reported IOP is not a simple average of the six measurements, nor an average after excluding the highest and lowest measurements as suggested previously.²⁸ Because of the ambiguity in how the tonometer-reported IOP was calculated, we used a second method to obtain IOP by manually calculating the average of the first five rebound measurements as presented on the tonometer display after each rebound (the sixth rebound measurement was not actually displayed, but rather was replaced with the tonometer-reported IOP described above). We referred to this as the "manually-calculated IOP." When applied to the same calibration data, both methods exhibited a linear relationship with manometric IOP, but the manually-calculated IOP yielded a larger R^2 value (0.96 vs. 0.91), indicating greater linearity and a smaller root-mean-squared error in relation to the true manometric pressure (2.2 vs. 1.5 mm Hg). For this reason, we used the manually-calculated IOP for all further IOP measurements in this study.

For each strain, the tonometric pressure (IOP_t) was expressed as a linear function of the true manometric pressure (IOP_m) as determined by linear regression based on the following relationship:

FIGURE 2. A typical perfusion tracing showing the flow rate (solid tracing, left axis) and pressure (dashed tracing, right axis) as a function of time for one enucleated eye of a C57BL/6J mouse. The pressure increases in steps of 4, 8, 15, and 25 mm Hg, while the flow rate increases in proportion to the increase in pressure. The spikes in flow rate are due to rapid adjustments of the syringe pump as it attempts to maintain the desired pressure. The average flow rate at each pressure step is calculated by averaging over at least 10 minutes of data, excluding the spike values.

$$IOP_t = \alpha \times IOP_m + \beta \quad (1)$$

where the slope (α) and intercept (β) values are given in Table 1. Following previous studies,^{29,30} there was no statistical difference in the parameters of the linear regression between strains ($P > 0.48$, 1-way ANOVA). Data from all strains, therefore, were lumped together to yield a single linear regression (Fig. 1, Table 1). All tonometric IOP values reported in this study were corrected by the following relationship:

$$IOP = 1.0 \times IOP_t + 0.1 \quad (2)$$

where the units are in mm Hg and IOP_t is the “manually-calculated IOP” described above. This correction amounts to a small increase of approximately 1% at physiologic IOP with the tonometer tending to slightly underestimate the true pressure.

To investigate the effects of anesthesia, IOP measurements were attempted in 10 conscious mice of each strain and again in the same mice under anesthesia within one or two days. For conscious measurements, mice were placed in a customized restraining device and allowed to settle for 3 minutes before tonometry in the right eye only. Despite repeated training attempts, conscious IOP measurements were possible in only 21 mice (6–8 per strain) because either the mice did not remain still or IOP values were unreasonably large (>20 mm Hg). For anesthetized measurements, mice were exposed to 4% isoflurane (IsoFlo; Abbott Animal Health, Abbott Park, IL) and 2% oxygen for 3 minutes in a sealed box, followed by 3.5% isoflurane and 0.7% oxygen via a nose cone for 2 to 3 minutes. The IOP was measured in the right eye between 5 and 6 minutes after the initial exposure to anesthesia. Previous studies reveal that anesthesia lowers IOP in mice,²⁴ and that IOP measurements should be made within minutes following the onset of anesthesia to minimize the IOP-lowering effect.⁹ Animals then were allowed to recover under a steady flow of oxygen without isoflurane.

Conventional Outflow Facility (C) Measurements

Outflow facility was measured in ex vivo mouse eyes using a computer-controlled perfusion system modified from previous studies^{7,16,18,31} to allow perfusion while the eye was submerged in a heated bath of isotonic saline (see below). On a typical experimental day, one eye was perfused from each strain, and the order of perfusion was randomized. Eyes were enucleated within 15 minutes of death by cervical dislocation, and eyes were stored in PBS at 4°C for no longer than 6 hours before perfusion. All mice were euthanized and their eyes enucleated at approximately the same time. Facility was

measured in only one eye per mouse, typically the right eye ($N = 20$ mice) unless an unsuccessful perfusion required the left eye to be perfused ($N = 8$). Perfusion was unsuccessful in both eyes of two mice.

For perfusion, the eye was mounted in a plastic tube trimmed from a 0.2 mL standard centrifuge tube that was filled with gauze, with the posterior sclera affixed to the tube using cyanoacrylate adhesive. Eyes were immersed to the limbus in a PBS bath and covered with tissue paper to provide thorough hydration. The PBS bath was heated indirectly with a custom-built temperature-controller to maintain bath temperature in the physiologic range of $35^\circ\text{C} \pm 1^\circ\text{C}$. The eye was cannulated using a 33-gauge needle that was inserted into the anterior chamber under a stereomicroscope using a micromanipulator. The needle was connected via rigid pressure tubing to a pressure transducer (142PC01G; Honeywell, Columbus, OH) and a 25 μL syringe (Gastight; Hamilton, Reno, NV) placed on a computer-controlled syringe pump (PhD Ultra; Harvard Apparatus, Holliston, MA). Custom-written software¹⁸ was used to maintain IOP at a user-defined pressure by adjusting the variable flow rate of the syringe-pump. All eyes were perfused with Dulbecco's PBS including divalent cations and 5.5 mM glucose passed through a 0.22- μm filter (DBG).

To measure C , eyes were perfused at sequential pressure steps of 4, 8, 15, and 25 mm Hg, typically 20 minutes at each step to allow at least 10 minutes of steady flow rate data (Fig. 2). The average flow rate (F) at each pressure step (P_p) then was fit to a simplified version of Goldmann's equation:

$$F = C \times P_p + F_u \quad (3)$$

where episcleral venous pressure and aqueous humor production are assumed to be zero for an enucleated eye. In Equation 3, perfusion pressure is defined as P_p to avoid confusion with IOP as measured in vivo. According to Equation 3, C is equal to the slope of the least-squares linear regression of F versus P_p measured during perfusion, and F_u is the zero-pressure intercept that often is interpreted (correctly or incorrectly) as unconventional or pressure-independent outflow. Equation 3 assumes that C and F_u are independent of P_p , and that F reaches a steady state at each pressure step. For a perfusion to be acceptable, the flow rate must achieve stable value, as indicated by a deviation of less than approximately 10%, in at least three of the four pressure steps. According to these criteria, C was measured successfully in 28 of 30 mice (9–10 per strain). Of the 28 mice where C was measured, 14 mice also had measurements of conscious IOP without anesthesia (3–6 per strain). Of these 14 mice, 11 mice had facility measured in the right eye (the same eye used for IOP

FIGURE 3. IOP measured in conscious (*white*) and anesthetized (*black*) mice from BALB/cJ ($N = 8$ and 10), C57BL/6J ($N = 6$ and 10), and CBA/J ($N = 7$ and 10) strains. The conscious mice were a subset of the anesthetized mice, and were fewer because of difficulties obtaining IOP measurements in conscious animals (see text and Table 2 for further details). IOP was corrected as described in Equation 2. *Error bars:* SD.

measurements) and three mice had facility measured in the left eye.

Statistical Analysis

The IOP was analyzed using a linear mixed model (LMM) to test for statistical differences between strains with and without anesthesia (SPSS Software; IBM, Armonk, NY). Two fixed effects were included in the LMM (strain and anesthesia) with IOP as the dependent variable. The maximum likelihood method was used to estimate the solutions to the LMM equations. Using a full-factorial design, the LMM analysis yielded three P values (corresponding to the probability of a type I error) describing the influence of strain, anesthesia, and interactions between strain and anesthesia on IOP. If a significant influence was detected in any one factor, the software automatically performed a post hoc Bonferroni test to evaluate pairwise differences between any two groups. To investigate further the effect of anesthesia in each strain, conscious and anesthetized IOP measurements from the same eye were compared using a paired Student's t -test (6–8 eyes per strain). To determine whether IOP differences between strains were masked by anesthesia, IOP was compared between strains for anesthetized mice only ($N = 30$) using a 1-way ANOVA.

Differences in C , F_w , α , and β between strains were analyzed using a 1-way ANOVA, followed by a post hoc Bonferroni test for detecting pairwise differences between any two strains. Linear regression analysis was performed using Excel (Microsoft Corporation, Redmond, WA). A bivariate correlation analysis was performed to evaluate whether there was a statistically significant correlation between IOP and $1/C$. More specifically, we computed the Pearson's product-moment correlation coefficient, and tested whether this quantity was significantly different from 0 using a 2-tailed t -test (SPSS; IBM).

Data points were considered outliers in the linear regression if the Cook's distance was larger than 1.0 or if the probability of the studentized deleted residual was lower than $0.05/N$, where N was taken as the number of data points used for the regression.³² Data points were considered outliers from a population based on the Dixon's Q-test with a 95% confidence threshold.³³ Outlier analysis was performed separately on C and $1/C$. The significance threshold was taken as 0.05 divided by the number of comparisons (e.g., 0.016 if comparing across three strains, per Bonferroni's correction).

RESULTS

IOP Measurements

The IOP was significantly different between strains (Fig. 3, $P = 0.001$, LMM). Consistent with prior reports,^{23,24} IOP was lowest for BALB/cJ and highest for CBA/J, with an intermediate value of IOP for C57BL/6J (Table 2). According to a post hoc Bonferroni test, IOP was statistically different between BALB/cJ versus CBA/J, but not between BALB/cJ versus C57BL/6J or C57BL/6J versus CBA/J. Compared to IOP in conscious mice, anesthesia caused a reduction in IOP (Fig. 3, Table 2; $P = 0.046$, LMM) that eliminated any detectable differences between strains ($P = 0.260$, 1-way ANOVA). Comparing IOP within individual eyes with and without anesthesia, the IOP reduction was significant for CBA/J ($26.0 \pm 18.3\%$, $P = 0.01$, paired Student's t -test with Bonferroni correction), but smaller or insignificant for C57BL/6J ($8.3 \pm 11.0\%$, $P = 0.14$) and BALB/cJ ($-0.9 \pm 29.1\%$, $P = 0.86$). These results were consistent with prior reports^{23,24} and demonstrated that anesthesia has an IOP-lowering effect that varies between strains or that has a stronger effect on mice that have a larger baseline IOP.

Conventional Outflow Facility (C) Measurements

Measurements of C were significantly different between strains ($P = 0.05$, 1-way ANOVA), as indicated by different slopes of the flow rate versus pressure relationship (Fig. 4). A post hoc Bonferroni test revealed that C was significantly larger in BALB/cJ compared to CBA/J ($P = 0.049$), with C57BL/6J having an intermediate value of C (Table 2). Importantly, the ranking of C between strains was opposite to the ranking of IOP, suggesting that strains that had a higher conscious IOP also had a lower C . There was no difference in the intercept of the flow-versus-pressure relationship (F_w) between strains ($P = 0.363$, 1-way ANOVA).

To investigate the relationship between IOP and C , conscious IOP was plotted against outflow resistance (the mathematical inverse of C , or $1/C$) as measured within individual mice (Fig. 5A, see Discussion for rationale). The relationship between IOP and $1/C$ was highly significant ($P = 0.0003$, 2-sided test for bivariate correlation, SPSS; IBM), demonstrating that larger conscious IOP was associated with increased outflow resistance. One data point (filled triangle in Fig. 5A) was found to be an outlier (probability of studentized

TABLE 2. IOP, C , Conventional Outflow Resistance (R), and F_w for Each Murine Strain (Mean $\pm$ SD)

Strain	IOP, mm Hg		C , $\mu\text{L}/\text{min}/\text{mm Hg}$	R , mm Hg/ $\mu\text{L}/\text{min}$	F_w , $\mu\text{L}/\text{min}$
	Conscious	Anesthetized			
BALB/cJ	10.6 ± 1.8 , $N = 8$	10.1 ± 2.3 , $N = 10$	0.0164 ± 0.0059 , $N = 10$	62.1 ± 18.2 , $N = 9^*$	-0.029 ± 0.041 , $N = 10$
C57BL/6J	12.3 ± 1.0 , $N = 6$	11.5 ± 2.4 , $N = 10$	0.0147 ± 0.0029 , $N = 9$	71.0 ± 16.6 , $N = 9$	-0.031 ± 0.032 , $N = 9$
CBA/J	14.5 ± 0.9 , $N = 7$	12.1 ± 3.2 , $N = 10$	0.0113 ± 0.0031 , $N = 9$	96.6 ± 33.6 , $N = 9$	-0.010 ± 0.022 , $N = 9$

* One outlier excluded based on Dixon's Q-test.

FIGURE 4. The average flow rate at each pressure step for BALB/cJ ($N = 10$), C57BL/6J ($N = 9$), and CBA/J ($N = 9$). Trend lines are plotted as the slope (C) and intercept (F_0) averaged over all eyes that were perfused for each strain (data given in Table 2). Flow rate and perfusion pressure correspond to F and P_p as defined in equation 3. Error bars: SD.

deleted residual was $0.00132 \leq 0.05/N = 0.00357$, see Methods) and was excluded from the regression, but the regression remained significant even if the outlier was retained ($P = 0.006$). The R^2 value of the regression in Figure 5A indicated that 70% of the variation in IOP between individual mice from three different strains can be attributed to variation in outflow resistance (excluding the one outlier).

DISCUSSION

In our study, we provided evidence that differences in IOP between three genetically distinct strains of mice are attributable largely to differences in C . Our data showed that the strain having highest IOP (CBA/J) had the lowest outflow facility, and, conversely, the strain having the lowest IOP (BALB/cJ) had the highest outflow facility. The strain having an intermediate value of IOP (C57BL/6J) had an intermediate value of outflow facility. Regression analysis applied to individual mice from all three strains revealed a highly significant correlation between conscious IOP and conventional outflow resistance ($1/C$), with 70% of the variability in IOP being attributable to differences in conventional outflow resistance as predicted by Goldmann's equation.

Intraocular Pressure

Our IOP data showed clear differences among three inbred strains, in good agreement with prior reports. Using the data reported by Savinova et al.,²³ IOP for BALB/cJ, C57BL/6J, and CBA/J under general isoflurane anesthesia is approximately 11, 13, and 16 mm Hg, respectively (read from Fig. 1 of the report of Savinova et al.²³). Similarly, Wang et al.²⁴ reported IOP in conscious BALB/c, C57BL/6, and CBA as 10.6, 13.3, and 16.4 mm Hg. These values are in reasonable agreement with our conscious IOP measurements reported in Table 2. In regard to anesthesia, our results suggested that the IOP-lowering effect of isoflurane anesthesia varies between strains, with a larger IOP reduction observed in strains having a higher baseline IOP, consistent with prior reports.^{9,24} Importantly, these data demonstrated that IOP differences between strains can be masked by anesthesia, and, thus, the anesthetic regimen is an important consideration for future studies examining aqueous humor dynamics in mice.⁹

Our preliminary studies examined IOP in a fourth strain, CBA/CaJ, chosen based on prior reports²³ suggesting that CBA/CaJ mice exhibit a high baseline IOP (19.3 mm Hg). In our hands, however, CBA/CaJ mice exhibited an intermediate IOP of 11.7 ± 1.3 mm Hg ($N = 4$) that was not dissimilar from IOP in C57BL/6J (12.3 ± 1.0 mm Hg), and, therefore, we replaced CBA/CaJ with CBA/J. It is unclear why our IOP measurements in CBA/CaJ were lower than reported by Savinova et al.,²³ but this difference may be attributable to age because Savinova et al.²³ used mice between 6 and 12 months, compared to our study that measured IOP in mice between 3 and 4 months. Consistent with our results, another group also reported intermediate IOP values for CBA/CaJ mice as measured via telemetry (12.4 ± 2 mm Hg averaged over the entire light cycle from 6 AM to 6 PM), but age was not specified in that study.³⁴

Conventional Outflow Facility (C)

Our data showed that CBA/J mice that have a greater IOP also have a lower C , while BALB/cJ mice that have a lower IOP also have a greater C , relative to C57BL/6J mice. Therefore, differences in C can account for a portion of the differences in IOP between strains (see quantitative analysis below). Our measurements of C also are in good agreement with published values for C57BL/6J ($0.0105 \mu\text{L}/\text{min}/\text{mm Hg}^{35}$) and BALB/c(J) ($0.006\text{--}0.018 \mu\text{L}/\text{min}/\text{mm Hg}^{8,9}$), although we noted that the literature values are rather ambiguous because they span a rather large 3-fold range, which makes comparison difficult. To the best of our knowledge, there are no prior reports of C in CBA/J mice.

These data raised the interesting question of whether morphologic differences in the conventional outflow pathway between strains may underlie the differences in C . Savinova et al.²³ also examined this question in the context of strain-dependent differences in IOP, and found no obvious anatomic or pathologic differences in the drainage angle structures between strains. Other studies, however, have reported differences in gross ocular anatomy between strains, including anterior chamber depth, axial length, and corneal thickness.³⁶ We, ourselves, attempted a comparison of trabecular meshwork and Schlemm's canal between strains, but the histologic sections were of insufficient quality to allow a proper analysis.

FIGURE 5. (A) Conscious IOP versus conventional outflow resistance ($1/C$) measured within individual mice for BALB/cJ ($N = 6$), C57BL/6J ($N = 5$), and CBA/J ($N = 3$). These data include only those mice where conscious IOP and C were measured in the same individual. The trendline and equation represents the linear regression of IOP versus $1/C$ excluding the one outlier (indicated by the filled triangle, see Equation 5). The slope of the regression provides an estimate of the conventional volumetric outflow rate ($F_c = 0.068 \pm 0.013 \mu\text{L}/\text{min}$, see Equation 5), while the intercept provides an estimate of EVP ($7.1 \pm 1.0 \text{ mm Hg}$), assuming constant values for all mice. (B) The mean conscious IOP ($\bar{IOP}$) versus mean outflow resistance ($\bar{R}$ = the mean value of $1/C$) measured for the entire set of BALB/cJ ($N = 8$ for $\bar{IOP}$ and $N = 9$ for $\bar{R}$, with one outlier excluded), C57BL/6J ($N = 6$ and 9), and CBA/J ($N = 7$ and 9). These data include all mice of each strain where conscious IOP and C were measured, not necessarily in the same individual. The trendline and equation represents the linear regression of $\bar{IOP}$ versus $\bar{R}$ using only three data points (one per strain, see Equation 6). The population-based estimates of F_c ($0.108 \pm 0.022 \mu\text{L}/\text{min}$) and EVP ($4.2 \pm 1.7 \text{ mm Hg}$) are not statistically different from the estimates based on individual eyes. Error bars: SD. See Discussion for further details.

It is interesting that the albino BALB/cJ strain exhibited the lowest IOP and highest outflow facility compared to the pigmented strains CBA/J and C57BL/6J. Pigment typically accumulates in the trabecular meshwork and phagocytosed pigment granules are observed commonly in trabecular meshwork cells.³⁷ Heavy pigmentation of the trabecular meshwork occurs in pigmentary dispersion syndrome, and excess pigment load may lead to acute obstruction of the trabecular meshwork.³⁸ Thus, it is possible that differences in pigmentation or the cellular response to pigment may underlie the differences in outflow facility and IOP between pigmented and nonpigmented strains. Future studies including a larger

number of strains would be needed to examine more thoroughly the relationship between pigmentation and outflow facility in mice.

It should be noted that our measurements of C were done *ex vivo* using enucleated eyes, while most other studies measured C *in vivo* under anesthesia. Following enucleation, one safely may assume that aqueous production is eliminated ($F_{in} = 0$) and the episcleral veins are open to the atmosphere (EVP = 0 mm Hg). Enucleated eyes, therefore, provide a somewhat more direct means to measure C without possible confounding effects of F_{in} or EVP that may influence *in vivo* measurements. However, we must assume that C measured in enucleated eyes is similar to C *in vivo*. This assumption often is taken for granted in eyes of larger species, such as humans and rabbits,³⁹ where measurements of C in enucleated eyes are consistent with *in vivo* measurements of C by tonography or intracameral perfusion under anesthesia. For mice, however, to our knowledge no studies have yet compared C directly between enucleated and *in vivo* eyes. However, Millar et al.⁸ have shown that C is unaffected by euthanization via anesthetic overdose in BALB/cJ mice perfused *in situ* without enucleation.

Somewhat surprisingly, values of C measured in our study were significantly larger than reported previously by our own group^{16,31} for enucleated C57BL/6 eyes (0.0062 – $0.0091 \mu\text{L}/\text{min}/\text{mm Hg}$). These differences likely were caused by differences in temperature and hydration of the eye during perfusion. In our current study, the eye was immersed in a bath of isotonic saline at physiologic temperature. However, in our previous studies,^{16,31} the eyes were perfused at room temperature outside of a bath, with the eye covered by tissue paper that was moistened with isotonic saline. In our earlier studies,^{16,31} the facility at physiologic temperature was estimated by multiplying the slope of the flow-versus-pressure relationship measured at room temperature by a factor (~ 1.4) proportional to the temperature-dependent change in perfusate viscosity.⁴⁰ However, it is possible that effects other than viscosity may influence outflow facility differences between room and physiologic temperature. Furthermore, the zero-pressure intercept of the flow-versus-pressure relationship (F_u in Equation 3) in this study was not significantly different from zero, in contrast with our previous studies,^{16,31} where this intercept was significantly larger than zero (0.035 – $0.157 \mu\text{L}/\text{min}$). This intercept often is interpreted as the quantity of outflow passing through the unconventional or pressure-independent pathway.^{16,31} It is important to recognize that this intercept is an extrapolated value that is estimated based on flow rate measurements at nonzero pressures, and measurements at higher pressures may have significant leverage on the estimate of F_u . Osmotic differences between the intraocular and extraocular compartments also may influence the zero-pressure intercept by driving flow in the absence of an applied pressure gradient. Moreover, if the apparent intercept is, indeed, so strongly dependent upon hydration, temperature, and/or osmolarity, then the accuracy and interpretation of the extrapolated intercept (often taken to represent unconventional outflow) may be drawn into question, at least based on studies in enucleated eyes. Ongoing studies are examining how temperature, hydration, and osmolarity affect C and F_u in an attempt to resolve this important issue.

Relationship Between IOP and C

Plotting IOP versus $1/C$ as shown in Figure 5A provides a physically meaningful interpretation, as can be seen using the modified Goldmann's equation:

$$F_{in} = C \times (IOP - EVP) + F_u \quad (4)$$

where F_{in} is the aqueous production rate, EVP is episcleral venous pressure, and F_u is the outflow rate through the unconventional or pressure-independent pathway. All parameters in Equation 4 apply to conscious mice. Solving Equation 4 for IOP yields:

$$IOP = \frac{F_c}{C} + EVP \quad (5)$$

where F_c is the outflow rate through the conventional pathway ($F_c = F_{in} - F_u$). Assuming that C measured in enucleated eyes represents C in vivo (see above), Goldmann's equation thereby predicts a linear relationship between IOP and conventional outflow resistance ($1/C$), with the slope of the relationship equal to F_c and the zero-IOP intercept equal to EVP . This method, therefore, allows indirect estimates of F_c and EVP based on measurements of C and IOP from a sample population of mice (or any species for that matter).

If we assume that F_c and EVP are constant between C57BL/6J, BALB/cJ, and CBA/J mice, then the regression in Figure 5A predicts (excluding the outlier) that $F_c = 0.068 \pm 0.044$ $\mu\text{L}/\text{min}$ and $EVP = 7.1 \pm 3.2$ mm Hg (mean $\pm$ SD). Whether F_c and EVP truly are constant between strains is an open question. However, the strong agreement between Equation 5 and our data ($R^2 = 0.70$, $N = 13$ mice) suggests that F_c and EVP are reasonably consistent between C57BL/6, BALB/cJ, and CBA/J strains, or that any variation in F_c and EVP between strains has relatively minor influence ($\leq 30\%$) on IOP compared to the principal dependence of IOP on $1/C$. We point out that a portion of the remaining 30% influence is likely attributable to experimental uncertainty in the measurements themselves. The value of EVP estimated by linear regression (7.1 mm Hg) agrees fairly well, but tends to be slightly higher than published values from the same strains using the blood-reflux technique²¹ (compare to 5.4 mm Hg for BALB/cJ⁸ and 6.3 mm Hg for C57BL/6J³⁵) that requires anesthesia where EVP may be depressed artificially. The value of F_c estimated by linear regression (0.068 $\mu\text{L}/\text{min}$) also fits within the published range for F_c that varies 3-fold between strains (0.032 $\mu\text{L}/\text{min}$ in NIH Swiss White,²¹ 0.060 $\mu\text{L}/\text{min}$ for C57BL/6,³⁵ and 0.111 $\mu\text{L}/\text{min}$ for BALB/cJ⁸), which may be related to methodologic differences between studies or true physiologic differences between strains. It should be pointed out that F_c lumps effects of F_{in} and F_u , such that variations in F_{in} between strains may be masked by compensatory differences in F_u (or vice versa), and this approach, therefore, is unable to make inferences about F_{in} or F_u without additional data beyond C and IOP. We attempted to perform regression analysis on each strain individually, so as to estimate values of F_c and EVP for each strain, but the small numbers of mice (3–6 per strain) made this analysis unreliable.

As an alternative to the regression analysis of individual eyes presented above, one also may examine a population-based analysis to investigate whether differences in the mean value of $1/C$ can account for differences in mean conscious IOP between strains. One advantage of the population-based analysis is that it uses all of our data for $1/C$ and conscious IOP (35 mice), not just data where $1/C$ and conscious IOP were measured in the same mouse (14 mice). One disadvantage, however, is that the number of data points in the population analysis is limited to the number of strains included in the study. Because Goldmann's equation essentially is a statement of the conservation of aqueous humor mass that is strictly true only for individual eyes (and not populations of eyes), Goldmann's equation must be expressed in terms of population averages (or expected values). Taking the expected value

of Equation 5 and neglecting any covariance between $1/C$ and F_c yields:

$$\overline{IOP} = \overline{F_c} \times \overline{R} + \overline{EVP} \quad (6)$$

where the overbars indicate population averages for a given strain, and $\overline{R}$ represents the average conventional outflow resistance (average value of $1/C$, Table 2). Assuming that $\overline{F_c}$ and $\overline{EVP}$ are the same for all strains, then Equation 6 predicts a linear relationship between $\overline{IOP}$ and $\overline{R}$ with a slope equal to $\overline{F_c}$ and an intercept equal to $\overline{EVP}$. As shown in Figure 5B, $\overline{IOP}$ and $\overline{R}$ from the three strains do, indeed, exhibit a linear relationship with 96% of the variation in $\overline{IOP}$ between strains attributable to the variation in $\overline{R}$. However, with only three data points, there is only one degree of freedom for a linear fit, and, thus, the R^2 value (0.96) is almost certainly an overestimate of the true relationship between $\overline{IOP}$ and $\overline{R}$ (compare $R^2 = 0.70$ as predicted based on individual eyes; Fig. 5A). For the same reason, the predicted values of $\overline{F_c}$ (0.108 ± 0.022 $\mu\text{L}/\text{min}$) and $\overline{EVP}$ (4.2 ± 1.7 mm Hg) based on the population analysis are likely inaccurate, but nevertheless are not statistically different from the values predicted based on regression analysis of individual eyes ($P > 0.22$). More strains would be needed in the population-based analysis to improve these predictions.

In conclusion, our data suggest that differences in IOP between three genetically distinct strains of mice are attributable largely to differences in C , rather than to differences in aqueous inflow, episcleral venous pressure, or unconventional outflow. These data motivate further studies to identify the morphologic or genetic basis that underlie the strain-dependent differences in C to help illuminate the mechanisms of IOP regulation.

Acknowledgments

The authors thank the donors of National Glaucoma Research, and C. Ross Ethier (Georgia Institute of Technology) for thoughtful advice and comments.

Supported by grants from National Glaucoma Research, a Program of The BrightFocus Foundation (Formerly the American Health Assistance Foundation), the National Eye Institute (EY022359), Entente Cordiale Scholarship from the British Council, and the Santander Mobility Award from Santander Bank.

Disclosure: A. Boussommier-Calleja, None; D.R. Overby, None

References

- Grant WM. Clinical measurements of aqueous outflow. *AMA Arch Ophthalmol*. 1951;46:113–131.
- Smith RS, Zabaleta A, Savinova OV, John SW. The mouse anterior chamber angle and trabecular meshwork develop without cell death. *BMC Dev Biol*. 2001;1:3.
- Swaminathan SS, Oh DJ, Kang MH, et al. Secreted protein, acidic and rich in cysteine (SPARC)-null mice exhibit more uniform outflow. *Invest Ophthalmol Vis Sci*. 2013;54:2035–2047.
- Shepard AR, Millard JC, Pang IH, Jacobson N, Wang WH, Clark AF. Adenoviral gene transfer of active human transforming growth factor- β 2 elevates intraocular pressure and reduces outflow facility in rodent eyes. *Invest Ophthalmol Vis Sci*. 2010;51:2067–2076.
- Gottanka J, Chan D, Eichhorn M, Lütjen-Drecoll E, Ethier CR. Effects of TGF- β 2 in perfused human eyes. *Invest Ophthalmol Vis Sci*. 2004;45:153–158.
- Stamer WD, Read AT, Sumida GM, Ethier CR. Sphingosine-1-phosphate effects on the inner wall of Schlemm's canal and

- outflow facility in perfused human eyes. *Exp Eye Res.* 2009;89:980-988.
7. Boussoimmier-Calleja A, Bertrand J, Woodward DF, Ethier CR, Stamer WD, Overby DR. Pharmacologic manipulation of conventional outflow facility in ex vivo mouse eyes. *Invest Ophthalmol Vis Sci.* 2012;53:5838-5845.
 8. Millar JC, Clark AE, Pang IH. Assessment of aqueous humor dynamics in the mouse by a novel method of constant-flow infusion. *Invest Ophthalmol Vis Sci.* 2011;52:685-694.
 9. Camras LJ, Sufficool KE, Camras CB, Fan S, Liu H, Toris CB. Duration of anesthesia affects intraocular pressure, but not outflow facility in mice. *Curr Eye Res.* 2010;35:819-8127.
 10. Crowston JG, Aihara M, Lindsey JD, Weinreb RN. Effect of latanoprost on outflow facility in the mouse. *Invest Ophthalmol Vis Sci.* 2004;45:2240-2245.
 11. Saeki T, Ota T, Aihara M, Araie M. Effects of prostanoid EP agonists on mouse intraocular pressure. *Invest Ophthalmol Vis Sci.* 2009;50:2201-2208.
 12. Bahler CK, Howell KG, Hann CR, Fautsch MP, Johnson DH. Prostaglandins increase trabecular meshwork outflow facility in cultured human anterior segments. *Am J Ophthalmol.* 2008;145:114-119.
 13. Kumar S, Shah S, Deutsch ER, Tang HM, Danias J. Triamcinolone acetate decreases outflow facility in C57BL/6 mouse eyes. *Invest Ophthalmol Vis Sci.* 2013;54:1280-1287.
 14. Clark AE, Wilson K, De Kater AW, Allingham RR, McCartney MD. Dexamethasone-induced ocular hypertension in perfusion-cultured human eyes. *Invest Ophthalmol Vis Sci.* 1995;36:478-4789.
 15. Millard LH, Woodward DF, Stamer WD. The role of the prostaglandin EP4 receptor in the regulation of human outflow facility. *Invest Ophthalmol Vis Sci.* 2011;52:3506-3513.
 16. Lei Y, Overby DR, Boussoimmier-Calleja A, Stamer WD, Ethier CR. Outflow physiology of the mouse eye: pressure dependence and washout. *Invest Ophthalmol Vis Sci.* 2011;52:1865-1871.
 17. Johnson M, Chen A, Epstein DL, Kamm RD. The pressure and volume dependence of the rate of wash-out in the bovine eye. *Curr Eye Res.* 1991;10:373-375.
 18. Overby D, Gong H, Qiu G, Freddo TF, Johnson M. The mechanism of increasing outflow facility during washout in the bovine eye. *Invest Ophthalmol Vis Sci.* 2002;43:3455-3464.
 19. Scott PA, Overby DR, Freddo TF, Gong H. Comparative studies between species that do and do not exhibit the washout effect. *Exp Eye Res.* 2007;84:435-443.
 20. Erickson-Lamy K, Schroeder AM, Bassett-Chu S, Epstein DL. Absence of time-dependent facility increase ("washout") in the perfused enucleated human eye. *Invest Ophthalmol Vis Sci.* 1990;31:2384-2388.
 21. Aihara M, Lindsey J, Weinreb R. Aqueous Humor Dynamics in Mice. *Invest Ophthalmol Vis Sci.* 2003;44:5168-5173.
 22. Zhang Y, Davidson BR, Stamer WD, Barton JK, Lihua Y, Marmorstein AD. Enhanced inflow and outflow rates despite lower IOP in bestrophin-2-deficient mice. *Invest Ophthalmol Vis Sci.* 2009;50:765-770.
 23. Savinova OV, Sugiyama F, Martin JE, et al. Intraocular pressure in genetically distinct mice: an update and strain survey. *BMC Genet.* 2001;2:12.
 24. Wang WH, Millar JC, Pang IH, Wax MB, Clark AE. Noninvasive measurement of rodent intraocular pressure with a rebound tonometer. *Invest Ophthalmol Vis Sci.* 2005;46:4617-4621.
 25. Karp CL, Grupe A, Schadt E, et al. Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. *Nat Immunol.* 2000;1:221-226.
 26. Zhang D, Vetrivel L, Verkman AS. Aquaporin deletion in mice reduces intraocular pressure and aqueous fluid production. *J Gen Physiol.* 2002;119:561-569.
 27. Aihara M, Lindsey JD, Weinreb RN. Twenty-four-hour pattern of mouse intraocular pressure. *Exp Eye Res.* 2003;77:681-686.
 28. Pease ME, Hammond JC, Quigley HA. Manometric calibration and comparison of TonoLab and TonoPen tonometers in rats with experimental glaucoma and in normal mice. *J Glaucoma.* 2006;15:512-519.
 29. Pease ME, Cone FE, Gelman S, Son JL, Quigley HA. Calibration of the TonoLab tonometer in mice with spontaneous or experimental glaucoma. *Invest Ophthalmol Vis Sci.* 2011;52:858-864.
 30. Nissirios N, Goldblum D, Rohrer K, Mittag T, Danias J. Noninvasive determination of intraocular pressure (IOP) in nonsedated mice of 5 different inbred strains. *J Glaucoma.* 2007;16:57-61.
 31. Stamer WD, Lei Y, Boussoimmier-Calleja A, Overby DR, Ethier CR. eNOS, a pressure-dependent regulator of intraocular pressure. *Invest Ophthalmol Vis Sci.* 2011;52:9438-9444.
 32. Weisberg S. *Applied Linear Regression.* Hoboken, NJ: John Wiley & Sons; 1985.
 33. Dean RB, Dixon WJ. Simplified statistics for small numbers of observations. *Anal Chem.* 1951;23:636-638.
 34. Li R, Liu JHK. Telemetric monitoring of 24 h intraocular pressure in conscious and freely moving C57BL/6J and CBA/CaJ mice. *Mol Vis.* 2008;14:745-749.
 35. Lee YS, Tresguerres M, Hess K, et al. Regulation of anterior chamber drainage by bicarbonate-sensitive soluble adenylyl cyclase in the ciliary body. *J Biol Chem.* 2011;286:41353-41358.
 36. Puk O, Dalke C, Favor J, De Angelis MH, Graw J. Variations of eye size parameters among different strains of mice. *Mamm Genome.* 2006;17:851-857.
 37. Johnson DH. Does pigmentation affect the trabecular meshwork? *Arch Ophthalmol.* 1989;107:250-254.
 38. Epstein DL, Freddo TF, Anderson PJ, Patterson MM, Bassett-Chu S. Experimental obstruction to aqueous outflow by pigment particles in living monkeys. *Invest Ophthalmol Vis Sci.* 1986;27:387-395.
 39. Becker B, Constant MA. The facility of aqueous outflow; a comparison of tonography and perfusion measurements in vivo and in vitro. *AMA Arch Ophthalmol.* 1956;55:305-312.
 40. VanBuskirk, EM, Grant WM. Influence of temperature and the question of involvement of cellular metabolism in aqueous outflow. *Am J Ophthalmol.* 1974;77:565-572.