Pharmacologic Disruption of Schlemm's Canal Cells and Outflow Facility in Anterior Segments of Human Eyes

Cindy K. Bahler, Cheryl R. Hann, Michael P. Fautsch, and Douglas H. Johnson

Purpose. To determine the effect of disruption of Schlemm's canal cells on outflow facility. Pharmacologic agents that weaken the cytoskeleton or interfere with integrin binding may allow targeted disruption of the cells lining Schlemm's canal because of the transmural pressure gradient the cells face as aqueous passes into the canal.

Methods. Anterior segments of human eyes were placed in perfusion organ culture, and either single or sequential doses of H-7 or RGD peptide were added. Fellow eyes received vehicle or RGE peptide. Eyes were fixed and examined by light and electron microscopy.

Results. Both agents caused a partial loss of the endothelial lining of Schlemm's canal cells without disruption of trabecular cells in other regions. H-7 significantly increased outflow facility after single or sequential doses, with moderate cell loss of both the inner and outer wall canal cells: 20.0% ± 10.5% of the width of the canal versus 5.2% ± 3.7% in control meshworks ($P = 0.05$). No significant correlation between the amount of canal cell loss and outflow facility was found. RGD was associated with a variable loss of canal cells but did not change outflow facility.

Conclusions. Pharmacologic disruption of Schlemm's canal cells appears possible. H-7 increased outflow facility, causing a partial loss of the endothelial lining of Schlemm's canal. A simple relationship between canal cells and outflow facility was not found; canal cells probably interact with the extracellular matrix in influencing outflow facility. (*Invest Ophthalmol Vis Sci.* 2004;45:2246–2254) DOI:10.1167/iovs.03-0746

Classic thought on the site of aqueous outflow resistance has focused on the extracellular matrix of the trabecular meshwork near Schlemm's canal and on the endothelial cells of Schlemm's canal itself.[1–8] Evidence exists both to support and contradict the possibility that resistance is generated in each of these regions. Although the fluid channels within the extracellular matrix of the juxtacanalicular tissue are relatively small and tortuous, they are still large enough that the calculated outflow resistance of this region is 100-fold less than that actually measured in the eye.[2,3] If this region were filled with glycosaminoglycans, however, enough outflow resistance could be created to match that of the eye.[1] Schlemm's canal cells form a continuous endothelial barrier to aqueous entry into the canal and have been postulated to create outflow resistance.[4] The numerous intercellular and transcellular pores of this endothelial layer, however, cause the calculated resistance of this cellular barrier to be low.[5–7]

One approach to the question of cells versus extracellular matrix as the site of outflow resistance could involve removing the endothelial cells lining Schlemm's canal. If the canal cells were the primary site of resistance, disruption of this continuous cellular layer would greatly decrease outflow resistance. Although this task is impossible to do surgically, targeted disruption of these cells may be possible pharmacologically because of the pressure difference they face. Because of their position, the canal cells are interposed between the lower pressure of the lumen of Schlemm's canal and the higher aqueous pressure within the anterior chamber. If these canal cells were weakened with a cytoskeletal-disrupting agent, they may not be able to withstand this transmural pressure difference and could become disrupted or be washed off the basement membrane. Similarly, if the canal cell attachment to the underlying extracellular matrix were loosened, the cell could also be pushed off its position. In contrast, trabecular cells in other regions of the meshwork are surrounded by aqueous on all sides and face a much smaller pressure gradient. Despite weakening of their cytoskeleton or loosening of their attachments, non–canal cells may not be displaced from the meshwork because less pressure difference occurs across the cells.

We studied two agents with different cellular mechanisms of action in an attempt to disrupt Schlemm's canal's endothelial lining. H-7 (1-5-isoquinolinyl-sulfonyl-2-methylpiperazine; Sigma-Aldrich, St. Louis, MO) is a serine-threonine kinase inhibitor and decreases cellular actin-myosin contractility. It weakens the cytoskeleton and also loosens focal adhesions between the cell and the extracellular matrix.[9] H-7 reversibly decreased outflow resistance in the monkey, and also lowered outflow resistance in the whole porcine eye.[9,10] It has not been studied in the human eye. RGD peptide interferes with integrin binding of the cell to the extracellular matrix.[11,12] It decreased outflow resistance in porcine eyes (Kumar J, et al. *IOVS* 2000; 41:ARVO Abstract 4005), but did not change resistance in bovine eyes.[13] It has not been studied in human eyes.

Methods

Two studies were performed: (1) effect of H-7 or RGD peptide, and (2) determination of intraocular pressure after removal of the entire trabecular meshwork in an additional series of eyes. The rationale for removing meshwork was that if a lower pressure was found in the experimental eyes after removal of the entire meshwork than occurred after disruption of the canal cells with H-7, that difference in pressure must be caused by the remaining meshwork. This assumes that enough disruption of the canal endothelium occurs to eliminate its resistance. McEwan[14] has calculated that a 12-μm diameter hole is sufficient to drain aqueous; hence, if a greater loss of canal cells were present than the 12 μm diameter, it would suggest resistance was not in this layer.

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Supported in part by National Eye Institute Grant EY 07065, an unrestricted grant from Research to Prevent Blindness, Inc., and the Mayo Foundation.

Submitted for publication July 16, 2003; revised September 9, 2003 and January 27, 2004; accepted February 11, 2004.

Disclosure: **C.K. Bahler**, None; **C.R. Hann**, None; **M.P. Fautsch**, None; **D.H. Johnson**, None

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TABLE 1. Dosages and Responses to H-7 or RGD Peptide

	Dose (μM)	*n*	C_d/C_o Experimental	C_d/C_o Control
H-7	100	12	1.37*	1.02
	300	8†	1.23‡	1.00
RGD	50	5	1.16	1.10
	200	10	1.14	1.06
	500	5	1.00	1.03
	1000	6	0.94	0.94

* $P = 0.01$.
† All first received 100-μM dose.
‡ $P = 0.02$.

Culture Technique

Twenty-five pairs of fresh normal human eye bank eyes were studied (cultured 12 ± 5 hours after death; range: 3–21). The average age of the donor eyes was 70 ± 12 years (range: 33–86). No eyes had glaucoma, uveitis, pseudoexfoliation syndrome, or treatment with topical medications. The culture technique and intraocular pressure recording were similar to that described previously.[15] The eyes were obtained in accordance with the provisions of the Declaration of Helsinki for research involving human tissue.

Effect of H-7 or RGD

After an initial adaptation period in culture, one anterior segment of a pair was given H-7 or RGD peptide through an anterior chamber exchange, whereas that of the fellow control eye underwent an anterior chamber exchange with vehicle (H-7 group) or RGE peptide. The anterior chamber exchanges were performed by using a gravity-driven, constant-pressure method over a 5-minute period. Pressure data from either eye were not used during the first hour after the anterior chamber exchange. For eyes receiving sequential doses, the lowest concentrations were given first, followed no sooner than 24 hours later by the next higher dose.

H-7 was dissolved in the culture medium and administered in concentrations of 100 and 300 μM in either single or sequential doses. RGD peptide was given in concentrations of 50 to 1000 μM, using single-dose and multiple-dose regimens (Table 1). RGE peptide was used in the fellow eye as a control, at the same concentration as the RGD (G*RGD*SP, G*RGE*SP; Bachem, Inc, San Carlos, CA).

Outflow facility ($C = F/P$) was calculated every hour, beginning 3 hours before drug infusion and continuing for the duration of the culture. The drug effect on facility was calculated 8 hours after drug infusion. Anterior segments were subsequently fixed either by immersion in fixative or through anterior chamber perfusion, dividing each group equally between these methods. Fixative was 4% paraformaldehyde in 0.1 M phosphate buffer.

Removal of Entire Meshwork

After stable baseline pressures were obtained in eight pairs of anterior segments in perfusion organ culture (different eyes than used in the drug experiments), the anterior segments were removed from the culture dish. The entire meshwork was dissected and removed from the eye by making a shallow incision at Schwalbe's line and at the scleral spur, grasping the meshwork with a forceps, and gently teasing it from the eye. Anterior segments were returned to culture and intraocular pressures again recorded. Histologic sections from each quadrant of each eye were made to determine the completeness of meshwork removal. A preliminary experiment in four eyes determined that simple removal and replacement of the anterior segments from the culture dish (without removing meshwork) caused minimal change in intraocular pressure (increase of 13% ± 12%).

Histologic Examination

Wedges of tissue from the limbus were dissected from each quadrant. Two quadrants were prepared for transmission electron microscopy by dehydration in ascending concentrations of alcohol and embedding in epoxy resin. Two quadrants were prepared for scanning electron microscopy by dissection of wedges of tissue 2 mm wide and unroofing Schlemm's canal.[6] All cultures were evaluated by light and electron microscopy to assess the appearance of the trabecular cells, looking for evidence of toxicity.[15,16] In three pairs of anterior segments, cationic ferritin (10 mg/mL; Sigma-Aldrich) was given by anterior chamber exchange, followed by 30 minutes of perfusion, before fixation.[17]

Histologic Quantitation

Breaks in the cellular lining of the canal were quantified with both transmission and scanning electron microscopy. For transmission microscopy, two quadrants, 180° apart, were examined. The amount of breaks in the inner and outer wall of the canal lining was measured directly from electron micrographs at 1500× final magnification, and expressed as a percentage of the width of the canal (anterior to posterior aspect, appears as "length" of canal but is actually canal width when the circumferential nature of canal is considered). All pairs of anterior segments fixed by immersion (H7: 6 of 12 pairs; RGD: 8 of 13 pairs) were used for quantitation. Eyes fixed by perfusion were also examined, but did not undergo quantitation because of the possibility that perfusion fixation could have artifactually caused canal cell loss.

To look for washout or loss of the extracellular matrix underlying regions of canal cell loss, quantitation of the extracellular matrix immediately underlying the canal endothelial layer was performed. The length of the optically empty spaces immediately underlying the canal cells was measured and expressed relative to the width of the canal.[18] Note was made whether these spaces were in regions with loss of overlying canal cells. The length, rather than area, of the optically empty spaces was measured, as this measurement was correlated with outflow facility in previous studies.[19–21] Measurements of area of optically empty space in the juxtacanalicular region have not correlated with intraocular pressure nor the presence of glaucoma.[1–3]

For scanning microscopy, three pairs of anterior segments were examined from the H-7 group, chosen to represent eyes with low, medium, and high changes in facility after H-7. Two wedges of meshwork from each eye were examined by scanning the canal wall at 600× magnification, and the area of cell loss measured. Obvious dissection artifacts causing loss of cells were excluded. Higher power views were used as needed to confirm regions of cell loss. In the RGD group, four pairs were examined: two "responder" anterior segments and two "nonresponder" anterior segments.

Statistical Analysis

Drug effects were expressed as the outflow facility after drug infusion (C_d) at maximum time of change (8 hours for both H-7 and RGD responders) divided by the baseline facility (C_o) for each anterior segment.[9,10] Results from each pair of anterior segments were combined into a group mean for each concentration of drug. Microscopic measurements from each of the quadrants measured were combined to determine a mean value for the anterior segment. Data from each anterior segment was then combined to yield a group mean. Results are expressed as the mean ± SEM. Statistical significance was tested with either the paired two-tailed *t*-test or the nonparametric Wilcoxon signed rank test when data did not follow a Gaussian distribution.[22] Nonparametric methods have been used previously for meshwork and other studies by several investigators.[23–25] Correlations among cell loss, intraocular pressure, and facility of outflow were performed with Spearman's rank correlation.[22]

Calculations of hydraulic conductivity and flow resistance of the gaps in the canal cell lining used Sampson's law, $R = \Delta P/Q = 3 \cdot \text{viscosity}/r^3$, which considers flow across a thin membrane (the hole in the canal cell lining), where P is pressure, Q is flow, and r is the radius of the gap.[5] These calculations are based on the assumption that the changes found in the sampled quadrants were representative of the canal cell lining throughout the meshwork.

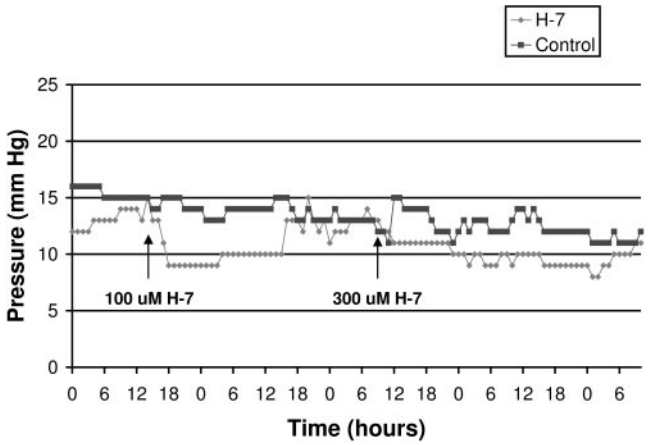


FIGURE 1. Intraocular pressure graph of the anterior segment receiving sequential doses of 100 μM and later 300 μM H-7 versus vehicle in fellow control anterior segment. The tracing covers a 4-day period: 0 is midnight. After the first H-7 dose, pressure decreased from 13 to 9 mm Hg, returning after 23 hours toward baseline. The second dose lowered pressure from 13 to 10 mm Hg. Control anterior segment pressure varied from 13 to 15 mm Hg through most of the period (52-year-old female).

RESULTS

Effect of H-7

H-7 and Facility. Outflow facility increased after the 100-μM dose by 37% ± 18% (mean ± SEM; $P = 0.01$), whereas the fellow control eyes receiving vehicle increased by only 2% ± 2%. Eight of 12 anterior segments had an increase in facility, whereas four had no change. Facility increased within the first hour after drug infusion and generally remained stable for approximately 8 to 12 hours, after which it returned toward baseline over the next 12 hours (Fig. 1). In the anterior segments with an increase in facility, facility later decreased toward the original baseline, returning to 82% ± 7% of baseline (Fig 1, for example; decrease in facility not statistically significant: $P = 0.17$). Anterior segments responding to H-7 did not differ in age, postmortem time to enucleation, or postmortem time to culture from eyes that did not respond.

Eight anterior segments received a subsequent dose of 300 μM (Table 1). This increased facility by 23% ± 3% ($P = 0.02$; fellow control no change). Three anterior segments responded to both the 100- and 300-μM dose, three pairs responded to the 300-μM dose only, one pair responded to the 100-μM dose but not the 300-μM dose, and one pair was nonresponsive to both doses. The effect on facility occurred within the first hour, but unlike the 100-μM dose, remained stable at the new level. Four pairs of anterior segments received a third dose of H-7 (300 μM), which did not cause a change in facility.

Ultrastructural Changes after H-7. Loss of Schlemm's canal cells was found in both the inner and outer walls of the canal after H-7 (Figs. 2, 3). Loss was patchy and variable and involved as much as 79% of the canal cell lining in one anterior segment. These changes occurred in both immersion- and perfusion-fixed eyes. Loss of canal cells appeared to involve two mechanisms: rounding of the cell with subsequent disintegration in place in the canal, leaving bare regions of extracellular matrix (Figs. 2A, 2B, 3A) or loss of the intact cell from its attachment to the underlying extracellular matrix (Figs. 2B, 2C). Cytoplasmic fingers, or processes, were present as the cells left the extracellular matrix (Figs. 2B, 3A, 3B). No obvious changes in the shape of the juxtacanalicular region or expansion of the inner wall region of the canal were found (Figs. 2A, 3A). Sabanay et al.[9] have postulated that this may occur in the monkey eye and may be responsible for changes in outflow facility. Scanning electron microscopy confirmed patchy regions of cell loss, with some regions showing bare basement membrane, whereas other regions had loss of both cells and basement membrane (Fig. 2C).

Trabecular cells in the other portions of the meshwork appeared intact, with no apparent cell loss by subjective examination. Cells remained attached to the trabecular lamellae, with no evidence of rounding up or loss of their usual horizontally spread configuration (Figs. 2A, 3A). Mitochondria and endoplasmic reticulum remained intact in these cells; no histologic evidence of toxicity was found. The overall configuration of the meshwork remained normal. The juxtacanalicular region did not expand, the inner and outer walls of the canal did not increase in length or area, and the lamellae remained intact and in usual proximity to each other.

The underlying extracellular matrix generally appeared intact, without disruption even in regions with overlying cell loss (Figs. 2A, 2B, 3B), although scattered regions of loss were probably present (Fig. 3).

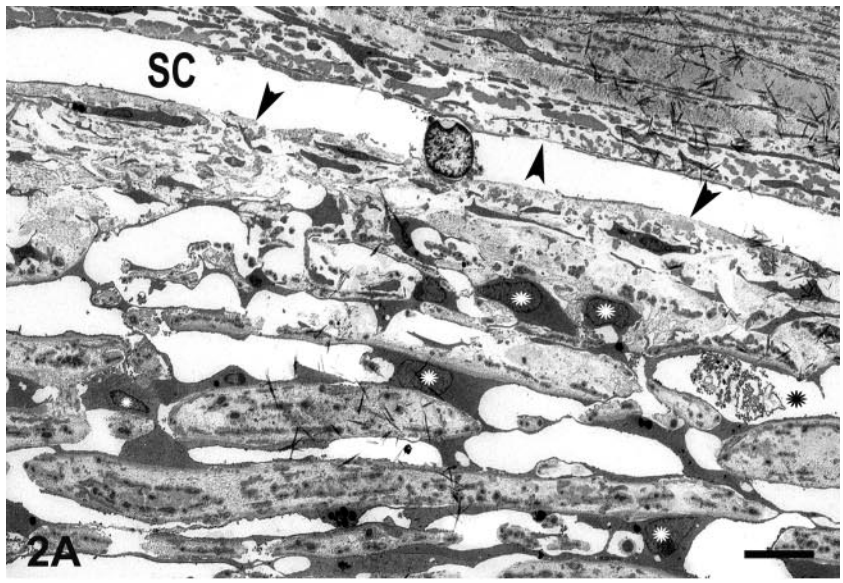
Quantitative Results. Quantitation revealed greater cell loss in experimental than control anterior segments in all six pairs that had quantitation performed (20.0% ± 10.5% vs. 5.2% ± 3.7%; $P = 0.05$). These six pairs were all fixed by immersion rather than perfusion fixation, as stated previously, to avoid artifactual loss of canal cells by perfusion. Of interest, one of the six pairs analyzed was a nonresponder to H7, and it had minimal cell loss in both the H7 and control meshworks: 1.0% ± 1.3% versus 0.0%. Scanning electron microscopy was similar to transmission electron microscopy in finding a greater loss of canal cell lining in experimental than control meshworks (14.2% ± 11.0% vs. 1.0% ± 0.4% loss), although the difference was not statistically significant because of the variability among tissue samples.

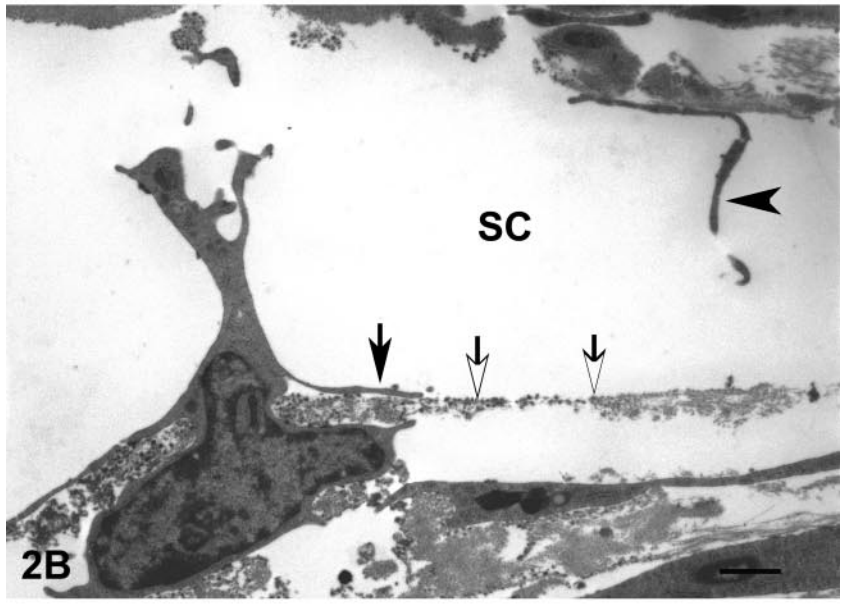
The number of gaps in the cell lining of the canal was greater in the experimental than control eyes: 12.0 ± 7.5 gaps/mm vs. 6.6 ± 4.8 gaps/mm, $P = 0.003$). The mean size of gaps in the canal lining was 4.4 ± 2.4 μm (range, 1.0–39.7) in experimental and 4.4 ± 3.4 μm (range, 1.0–23.8) in control eyes. No difference was found between inner and outer wall regions. The distribution of gap sizes was not Gaussian: 35% of gaps were 1.6 μm or smaller in size; the median was 8.5 μm, mode was 1.6 μm. This distribution was similar between experimental and control eyes. Calculations using Sampson's law indicate that these gaps were sufficient in size and number to eliminate fluid resistance of the canal lining cells. The calculated facility of outflow was 358 μL/min per mm Hg in experimental and 230 μL/min per mm Hg in control eyes—two orders of magnitude greater than the measured facility of outflow of 0.20 ± 0.10 μL/min per mm Hg in the experimental eyes.

Quantitation of the extracellular matrix revealed similar amounts of optically empty space adjacent to the canal cells among experimental and control meshworks. No statistically significant loss of extracellular matrix was found in regions with loss of overlying cells (Table 2).

Effect of RGD

RGD and Facility. Outflow facility did not significantly change after any dose of RGD (Table 1). Compared with control anterior segments, the maximum change in facility was an increase of 8% in the 200-μM dose (C_d/C_o, experimental versus control = 1.14 vs. 1.06; difference not statistically significant). Higher doses did not elicit greater changes in facility. Although the facility did not change for the combined group of anterior segments, a subgroup of 4 of the 13 had an increase in facility (mean increase of 41% ± 10%, versus fellow control





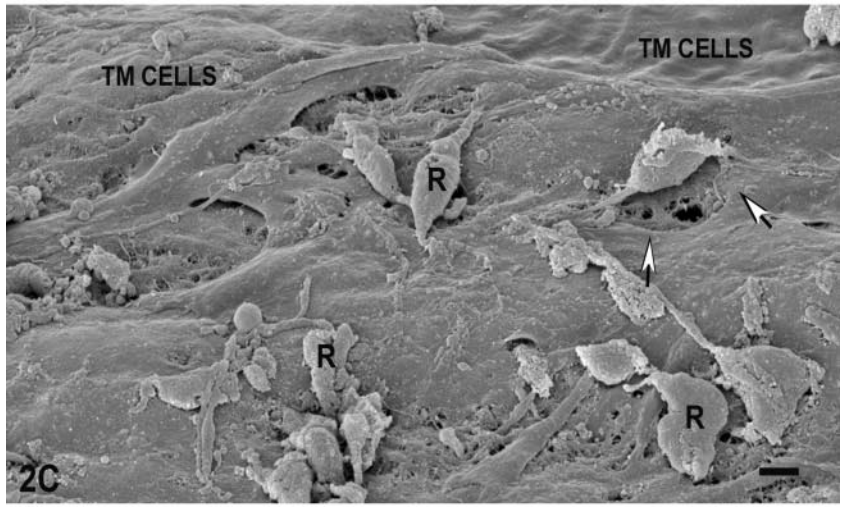


FIGURE 2. Scattered loss of canal cells after a single 100-μM dose H-7. Intraocular pressure dropped from 16 to 5 mm Hg, with final pressure of 9 mm Hg at the time of fixation 5 days later (immersion fixation, 70-year-old male). This anterior segment received cationic ferritin before fixation (10 mg/mL given by anterior chamber exchange followed by 30 minutes of perfusion before fixation). (**A**) Overview of meshwork. Rounded cell in the lumen of the canal. Scattered loss of canal cell lining is visible (*arrowheads*). Trabecular cells in other regions are intact and on lamellae (✸, *white*). Dead cell and cationic ferritin in the intertrabecular space (✸, *black*). (**B**) Loss of a canal inner wall cell with preservation of a thin layer of extracellular matrix (*open arrows*) labeled with cationic ferritin. Note the even and flat appearance of the matrix bordering the canal in the region where the inner wall cell had been (*filled arrow*). The outer wall cell is peeling off its position (*arrowhead*). (**C**) Scanning electron micrograph of the inside of the canal wall, showing intact trabecular meshwork cells adjacent to a region of rounded cells (R) and bare extracellular matrix. One rounded cell appeared to leave a gap in the underlying extracellular matrix (*open arrows*). SC, Schlemm's canal; TM, trabecular meshwork. Magnification: (**A**) ×1,500; (**B**) ×18,750; (**C**) ×1,300. Bar, (**A**, **C**) 10 μm; (**B**) 1 μm.

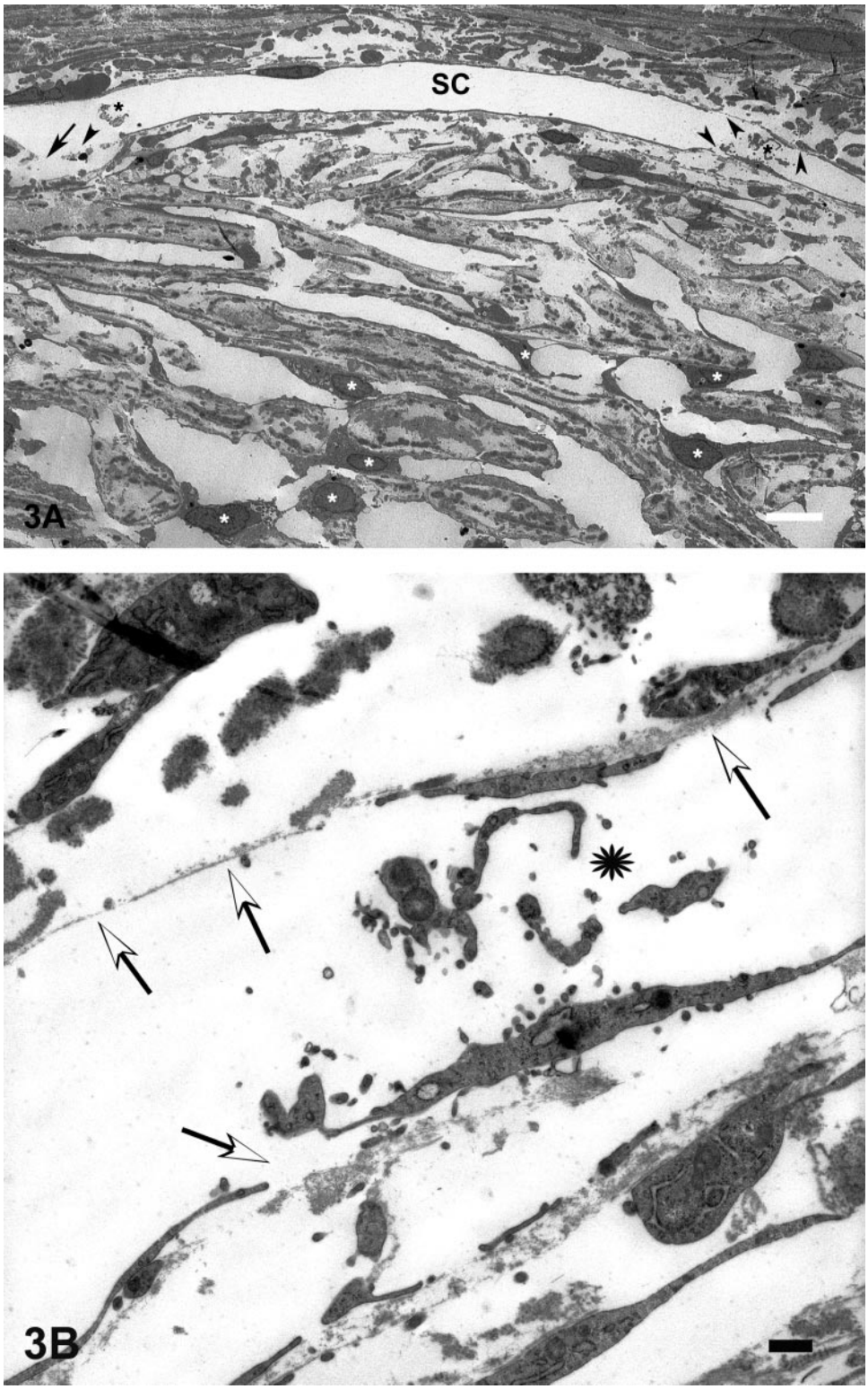


FIGURE 3. Scattered loss of canal cells after two doses H-7. Intraocular pressure decreased from 19 to 16 mm Hg after the first dose, then 1 week later a second dose decreased intraocular pressure from 14 to 11 mm Hg, and the pressure remained at this level until fixation (immersion fixation, 81-year-old male). (**A**) Overview of meshwork. Loss of canal inner and outer wall cells at each end of the canal (*arrowheads*). Shown are inner wall cells undergoing disruption (✶, *black*). Trabecular cells in the juxtacanalicular tissue and lamellar regions appeared intact (✶, *white*), supporting the idea that canal cells are "targeted." Some loss of extracellular matrix may be present (*arrow*). (**B**) Higher magnification of region from (**A**) showing disruption of inner and outer wall cells (✹). Cytoplasmic fingers and thin processes may have been due to H-7–associated weakening of the cytoskeleton. Cell loss leaving bare regions of extracellular matrix is also seen on the inner and outer wall (*open arrows*). SC, Schlemm's canal. Magnification: (**A**) ×1,500; (**B**) ×12,500. Bar: (**A**) 10 μm; (**B**) 1 μm.

eyes, 1.0% ± 0.1%; $P = 0.02$). Particular note was made of potential canal cell loss in this responder subgroup. In these anterior segments, lack of response to a smaller first dose did not preclude response to the subsequent, higher dose.

Ultrastructural Changes after RGD. Loss of Schlemm's canal cells was found in both the inner and outer walls of the canal after RGD, but was scattered and variable. The mechanism of cell loss appeared to differ between experimental and control eyes. In experimental eyes, canal cells appeared to "peel off" from their attachment to the underlying extracellular matrix (Figs. 4A, 4B), resulting in bare regions of extracellular matrix (Fig. 4). Quantitation revealed RGD-treated anterior segments had loss of canal cells for 19.0% ± 9.3% of the width of the canal versus 10.3% ± 5.8% in control anterior segments (difference not statistically significant). In the four responder eyes that had an increase in facility after RGD, cell loss was

TABLE 2. Measurements of Canal Cell Loss and Optically Empty Space Adjacent to Canal

		Loss of Canal Cell Lining (% of Canal)	Mean Gap Size in Canal Cell Lining (μm)	Optically Empty Space Adjacent to Canal (% of canal)	Optically Empty Space in Regions with Overlying Canal Cell Loss (% of Canal)
H-7	Exp	20.0 ± 10.5*	4.4 ± 2.8	14.7 ± 4.5	1.9 ± 0.7
	Con	5.2 ± 3.7	4.4 ± 3.4	9.9 ± 3.1	0.3 ± 0.2
RGD	Exp	19.0 ± 9.3	5.9 ± 5.1	12.1 ± 3.7	3.6 ± 2.3
	Con	10.3 ± 5.8	5.8 ± 4.2	13.5 ± 2.0	0.6 ± 0.6

All data are expressed as the mean ± SEM. Exp, experimental eyes; Con, control eyes.
* $P = 0.05$.

similar to the nonresponder group (18.2% ± 26.1% vs. 19.9% ± 29.7%). Scanning electron microscopy also did not find a statistically significant difference in cell gaps between responders and nonresponders.

Trabecular cells in the other portions of the meshwork appeared intact, with no apparent cell loss by subjective examination (Fig. 4A). Cells remained attached to the trabecular lamellae, with no evidence of rounding up or loss of their usual horizontally spread configuration.

In all eyes receiving RGD, the underlying extracellular matrix generally appeared intact, without disruption even in regions with overlying cell loss. Scanning electron microscopy revealed preservation of the underlying extracellular matrix in most areas of canal cell loss (Fig. 4C). Quantitation revealed similar amounts of optically empty space adjacent to the canal cells among experimental and control meshworks and no significant loss of extracellular matrix in regions with loss of overlying cells (Table 2).

Removal of Entire Meshwork

Intraocular pressures decreased from 17.1 ± 1.4 to 3.0 ± 0.5 mm Hg after meshwork removal ($P < 0.001$). Final pressures were independent of pressures before removal of the meshwork. The residual intraocular pressure is presumably from resistance in the collector channels and aqueous veins in the sclera, as described after trabeculotomy.[23,24] One eye had a final pressure of 14 mm Hg. Histologic examination revealed incomplete removal of the meshwork, with preservation of the canal, juxtacanalicular tissue, and outer corneoscleral lamellae. This eye was therefore deleted from the pressure analysis. Other eyes showed successful removal of the meshwork and canal.

Comparison of lowest intraocular pressures obtained in H-7 eyes to pressures in this experimental removal of the entire meshwork revealed significantly lower pressures after meshwork removal (H-7: 13.4 ± 2.1 mm Hg versus trabecular meshwork removal: 3.0 ± 0.5 mm Hg; $P = 0.002$).

Correlation between Cell Loss and Facility of Outflow

If the cells lining Schlemm's canal were responsible for most aqueous outflow resistance, loss of the cells should correlate with outflow resistance or its inverse, outflow facility. No correlations were found between cell loss and change in facility, final facility, or final intraocular pressure in the H-7 groups, either singly or combined (Fig. 5). The study had an 80% power to detect a correlation coefficient of 0.46.

DISCUSSION

H7 caused a reduction in intraocular pressure, associated with a partial loss of Schlemm's canal cells. Cytoskeletal-weakening agents, such as H-7 or cytochalasin D, appear to allow targeted disruption of Schlemm's canal cells.[21] Cells in other regions of the meshwork appeared intact and remained in position on the lamellae, suggesting toxicity was not associated with H-7.

Although the loss of the canal cells was presumably the mechanism of decrease of intraocular pressure, the relationship is not straightforward. If canal cell loss alone occurred, without disturbance of the underlying extracellular matrix, the amount of measured increase in outflow facility (37%) was greater than would have been predicted from the calculations of Bill and Svedbergh.[5] They suggested that the numerous transcellular pores in the canal cells would give this endothelial layer a high hydraulic conductivity, and should account for less than 10% of outflow resistance.[5] Our calculation of the expected facility of outflow after the loss of 20% of the canal cell lining predicted a much higher outflow facility than was found.

Several pieces of evidence suggest other factors besides simple loss of canal cells are involved. First, no correlation between cell loss and outflow facility was found. If canal cells had a significant effect on outflow facility, increasing cell loss should be accompanied by higher outflow facilities. Second, an equal amount of cell loss was present between the RGD and H-7 experimental groups, yet RGD did not increase facility, whereas H-7 did. Even in the select group of responder eyes in the RGD experiment, cell loss was not greater than in the nonresponder eyes. Overby et al.[13] also did not find RGD to cause a change in facility in bovine eyes at doses similar to those in the present study. We suspect that the different mechanisms of action between H-7 and RGD must be responsible for the different effects. RGD disrupts the cellular-integrin connections and may cause a "clean" cut between cell and extracellular matrix. In contrast, H-7 weakening of the cytoskeleton may cause the cell to disrupt some of the underlying extracellular matrix when it leaves its position in the canal.

Third, disruption of Schlemm's canal endothelium may affect outflow facility through loss of "funneling."[26] This hypothesis suggests that outflow resistance is modified by an interplay between the canal cells and their underlying extracellular matrix. In this scenario, the funneling of aqueous toward the small pores in the canal cells causes the extracellular matrix near the pores to have an effectively greater resistance than it would if no funneling of aqueous occurred.[26] Disruption of the canal cells would destroy the funneling effect and increase outflow facility more than predicted if only canal cell loss occurred.[5]

The meshwork and the juxtacanalicular region did not change conformation after H-7 or RGD. In living monkeys after H-7, the juxtacanalicular region expands and the inner wall of the canal stretches and moves into the canal lumen.[7] This difference between monkey and human response to H-7 probably relates to the extensive network of tendons and connecting fibrils present in the human meshwork. These tendons originate in the ciliary body and scleral spur, insert into the juxtacanalicular region and canal wall, and serve to anchor these regions and prevent collapse of the canal. The lack of

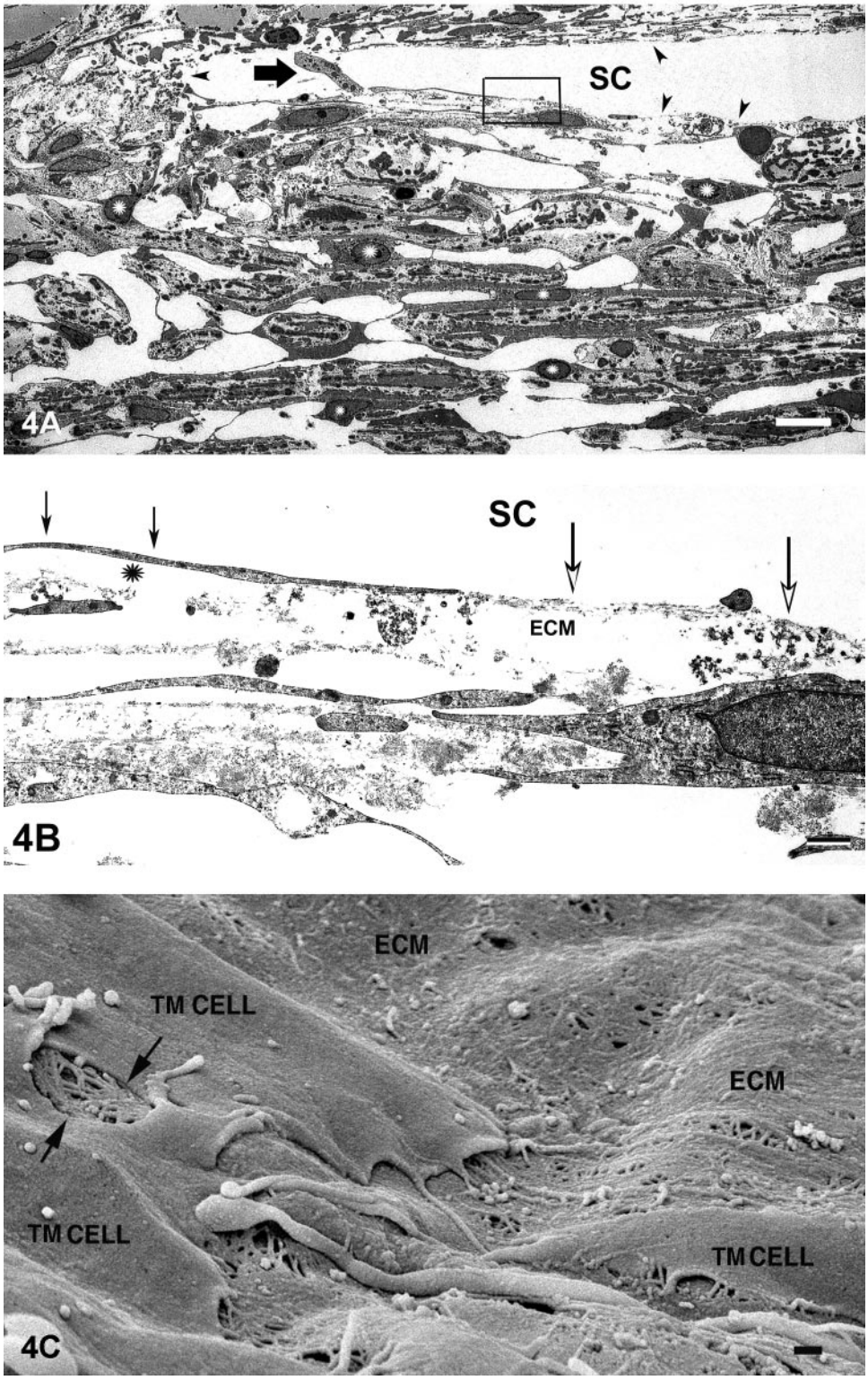


FIGURE 4. Trabecular meshwork of anterior segment receiving RGD. No significant change in outflow facility occurred after three doses (200, 500, and 1000 μM). Final intraocular pressure, 20 mm Hg (immersion fixation: 72-year-old female). (**A**) Loss of cells from Schlemm's canal lining is visible on both inner and outer walls (*arrowheads*). Intact canal cell lifting off extracellular matrix is visible (*arrow*). Remainder of trabecular cells appear healthy (✦, *white*). (**B**) Higher power magnification of (**A**). Inner wall cell covering is partially intact (*filled arrows*), and the underlying extracellular matrix appears intact (*open arrows*). (✸) Separation of an inner wall cell from the underlying extracellular matrix. (**C**) Scanning electron micrograph of inside of canal wall, same eye. Three inner wall cells remain, separated by regions of bare extracellular matrix (ECM). SC, Schlemm's canal. Magnification: (**A**) ×1,650; (**B**) ×5,000; (**C**) ×10,000. Bar: (**A**) 10 μm; (**B**, **C**) 1 μm.

these tendons in bovine eyes may explain the "washout phenomena," or progressive decrease in outflow resistance during anterior segment perfusion, in bovine eyes.[13,27] The lack of these tendons in monkey eyes may explain the occurrence of "washout" in the monkey eye.[7,27]

Loss of visible extracellular matrix did not accompany the canal cell loss, nor were increased optically empty spaces found in regions with loss of overlying canal cells (Table 2). This is in keeping with the presumption that components of the extracellular matrix not visible with conventional electron microscopy may be present.[1,28] Loss or changes in these soluble components of the extracellular matrix, not visible with conventional electron microscopy, may have occurred. Quick-freeze/deep-etch, an alternate method of processing and viewing tissue at the ultrastructural level, reveals a more elaborate and complex basement membrane than seen with conventional transmission electron microscopy. Although this technique preserves proteoglycans, glycosaminoglycans are not

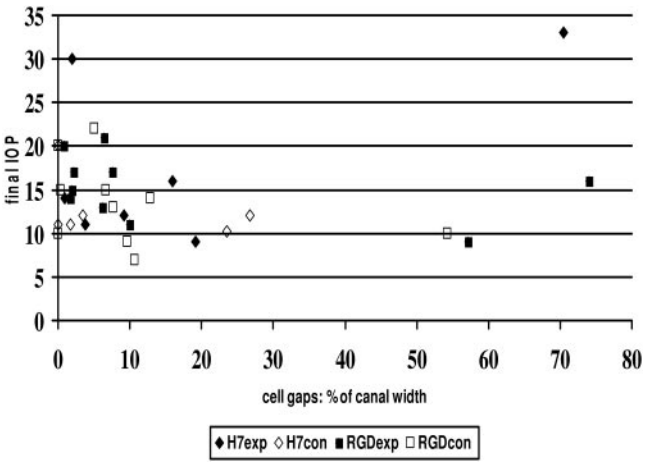


FIGURE 5. Scatterplot of canal cell loss as a percentage of the width of the canal and final intraocular pressure at time of fixation revealed no obvious relationship. Spearman correlation coefficient: −0.24 (P = 0.19, not statistically significant).

visualized.[28] In monolayer culture studies, disruption of actin may lead to a parallel disruption of the fibrillar fibronectin matrix outside the cell.[29] On detachment of the cells, fibronectin may contract to only one fourth of its stretched length.[30] In contrast to H-7's effect on actin, RGD peptide disrupts integrin connections of the cell to the extracellular matrix and may not cause as much disturbance to the underlying extracellular matrix. We specifically chose these two agents, with different mechanisms of action, to explore this idea. The lack of facility change despite cell loss similar in amount to that in the H-7 group supports this idea, as does the lack of effect of RGD on facility in the bovine eye.[13]

Can the amount of outflow resistance contributed by the canal cells be determined from these data? Two alternatives exist. If loss of 20% of the cell lining of the canal in the H-7 group were present homogeneously throughout the circumference of the canal, outflow resistance of the canal cells would effectively be eliminated, as predicted by our calculations using Sampson's law. In this scenario, fluid would preferentially flow through these holes in the canal lining. The outflow resistance remaining at the end of the experiments would thus be that contributed by the remaining meshwork. In the H-7 eyes the 37% change in facility would represent the amount of canal cell resistance, combined with any changes in the extracellular matrix. The difference between the lowest pressure in the H-7 group (13.4 mm Hg, R = 5.36 mm Hg/μL per minute) and the pressure resulting after removal of the entire meshwork (3.0 mm Hg, R = 1.2 mm Hg/μL per minute), represents the amount of resistance of the remaining meshwork (R = 4.16 mm Hg/μL per minute; or C = 0.24 μL/min per mm Hg).

The second alternative could be that the canal cell loss may not have eliminated the resistance of the entire canal cell lining. This could occur if the meshwork had segmental flow pathways, or channels, with limited lateral flow between regions with and without holes in the canal cell lining. In this case, a hole in the canal cell lining would not drain a widespread region of the upstream meshwork, but rather a more narrow channel. If this is correct, the canal cells provide a higher resistance than suggested herein. Against the idea is the lack of a significant correlation between canal cell loss and outflow facility (Fig. 5).

In summary, this study suggests that the cells lining Schlemm's canal play a greater role in outflow facility than previously assumed, but still do not account for most outflow resistance. Because the intertrabecular spaces are large enough that they should not create significant outflow resistance,[1] we speculate that the resistance of the meshwork remaining after canal cell loss is caused by the extracellular matrix of the juxtacanalicular region. We hypothesize that the resistance of the outflow pathway comprises a basic level of resistance provided by the extracellular matrix, with additional resistance and modulation of resistance created by the canal cells. This supports the theoretic concept of "funneling" as proposed by Johnson et al.[26]

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