Cationic Ferritin and Segmental Flow through the Trabecular Meshwork

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

Purpose. To determine whether segmental labeling by the tracer molecule cationic ferritin (CF) is indicative of preferential patterns of fluid flow in the trabecular meshwork or of differences in cell and extracellular matrix properties. Nonlabeled regions could indicate no fluid entering that area, insufficient perfusion time, or that the cells and extracellular matrix differ in that region and cannot bind CF.

Methods. Six whole eyes (three normal and three with pseudoexfoliation [PEX]) syndrome were perfused with CF for 30 minutes to 4 hours. Wedges of trabecular meshwork were dissected and some wedges immediately fixed. Adjacent wedges were placed in a CF bath before fixation. Transmission electron microscopy was used to analyze CF labeling.

Results. CF increased in the trabecular meshwork with increasing perfusion time. At 30 minutes, CF labeled mainly the uveal and corneoscleral regions. By 4 hours, CF was found diffusely through the meshwork, although a few isolated nonlabeled areas were still present. Wedges immersed in the CF bath showed fewer nonlabeled regions at all time points. Clumps of PEX material labeled more heavily in the periphery than the center, suggesting the clumps were less permeable than surrounding regions. PEX eyes otherwise had similar labeling patterns.

Conclusions. Segmental labeling with CF implies regions of preferential flow exist in the meshwork. With increasing perfusion time, there were fewer nonlabeled regions. CF labeling of most regions of bath-immersed tissue suggests that nonlabeled regions do not differ in the characteristics of the cells, but rather that CF does not reach these regions. (*Invest Ophthalmol Vis Sci.* 2005;46:1–7) DOI:10.1167/iovs.04-0800

As aqueous humor traverses the trabecular meshwork (TM) to reach Schlemm's canal (SC), it flows around collagenous lamellae that form interconnected aqueous spaces lined by trabecular cells. These aqueous spaces decrease in size as they approach the canal. The aqueous then passes through the basement membrane of SC endothelial cells and enters the canal through giant vacuoles and intercellular routes. Previous studies suggest that aqueous flow throughout the TM may be segmental. Clinically, segmental pigmentation is often observed during gonioscopy. A histologic study found that pigmented regions correspond to the locations of collector channels (Tanchel NA, et al. *IOVS* 1984;25:ARVO Abstract 7). A study of giant vacuoles found they were grouped near collector channels, indicating a greater pressure gradient and presumed aqueous flow in these regions.[1] An ultrastructural study in glaucomatous eyes concluded that preferential flow pathways probably exist, noting that pigmentation of the trabecular cells on the lamellae was more common in regions with normal lamellae and wide intertrabecular spaces than in regions with thickened, fused lamellae and narrow or absent intertrabecular spaces.[2]

Tracer studies also suggest that variations in flow occur within the circumference of the meshwork. A variety of tracers have been studied, including latex beads,[3] thorium dioxide (Thorotrast),[4,5] colloidal gold,[6] and cationic ferritin (CF).[7–11] CF has been used most frequently because of its small size (12 nm), positive charge, and ability to bind negatively charged cell surfaces. In living monkey eyes, CF labeled all regions of the TM except the region under the operculum, a "dead end" region of the TM.[8] In normal human eyes, results have varied among studies. In one study homogeneous labeling was present in all regions of the TM,[9] whereas in a more recent study segmental labeling was noted among quadrants and also within a single histologic section.[10] In glaucomatous eyes, segmental labeling has been reported among quadrants and within single histologic sections, in contrast to the finding of homogeneous label in normal eyes.[9] Segmental flow or alterations in flow could also occur in secondary glaucoma. Accumulation of pseudoexfoliation (PEX) material in the meshwork can decrease the size of the aqueous pathways and limit access to Schlemm's canal.[12] Although this is the presumed mechanism of glaucoma caused by PEX, the permeability of PEX and its effect on fluid flow, are unknown. CF labeling could help investigate this question.

The purpose of the present study was to re-examine the question of segmental flow in both normal human and PEX eyes and also to determine why CF labeling may vary in the TM. Segmental CF labeling could be due to segmental flow patterns or to differences in the characteristics of the cell surface proteins and the composition of the extracellular matrix (ECM). We studied this with two methods: increasing CF–tissue contact time by increasing CF perfusion times and elimination of potential low-flow regions by immersion of tissue in a bath of CF. We conclude that segmental fluid flow occurs in the TM, rather than differences in cell and ECM characteristics. In addition, PEX material was less highly labeled than other extracellular materials, confirming the idea that it is relatively impermeable and can clog the outflow pathways.

Materials and Methods

Six whole human eyes (mean age, 76 ± 17 years; range, 66–90) were obtained at autopsy within 8 hours of death. Research adhered to the tenets of the Declaration of Helsinki. Three eyes were normal, and

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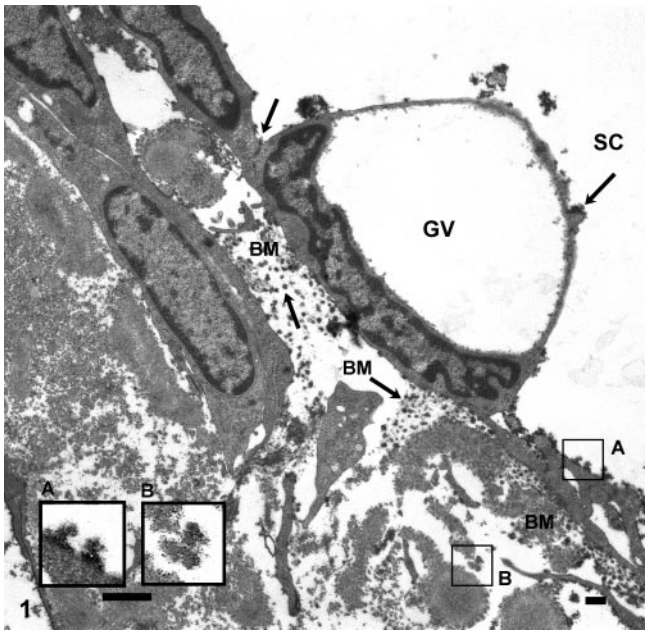

FIGURE 1. CF on the luminal surface of a Schlemm's canal (SC) cell, within the basement membrane (BM) and the endothelial cell junction (*arrows*). The labeling of the internal wall of the giant vacuole (GV) was weaker than the labeling of the luminal membrane of the same cell. *Inset A*: aggregations of CF on SC cell surface. *Inset B*: single particles associated with surface of sheath-tendon material. Scale bars, 0.25 μm.

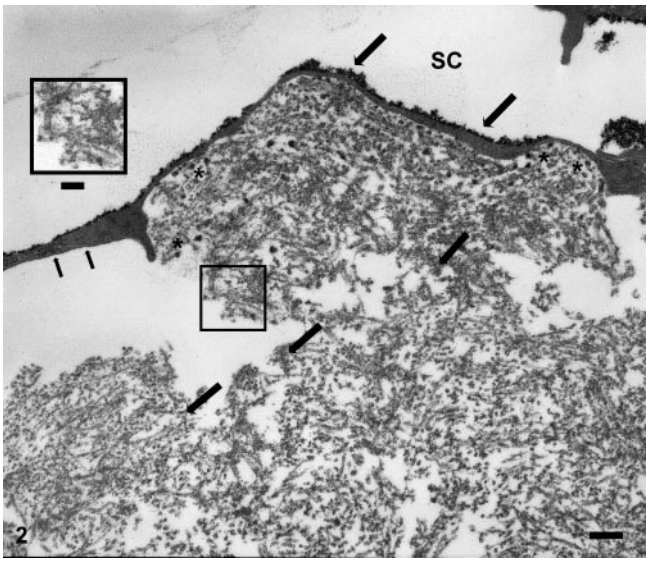

FIGURE 2. CF bath specimen of PEX eye. CF on luminal surface of Schlemm's canal (SC) cells and within fibrillar matrix of PEX material (*large arrows*). CF labeled mostly single particles, not large aggregates; clumps of CF were noted at the periphery of the PEX material (✶). *Small arrows*: regions with no CF label. *Inset*: single particles of CF within PEX material. Scale bars, 0.25 μm.

three eyes had PEX syndrome. PEX was documented from the history in two of three cases and discovered on ultrastructural examination in the third. In one case, timolol drops had been used. The PEX eyes were included to determine whether the PEX material would influence the pattern of fluid flow, as traced by CF label. At the start of the experiment, a 25-gauge needle was inserted into the posterior chamber, and eyes were perfused at 15 mm Hg for 60 minutes with Dulbecco's modified Eagle's medium (Mediatech Inc., Herndon, VA), containing antibiotics (10,000 U penicillin, 10 mg streptomycin, 25 mg amphotericin B, and 17 mg gentamicin [Sigma-Aldrich, St. Louis, MO], in 100 mL medium). This perfusion procedure established normal anterior chamber flow conditions.[1]

After the 1-hour perfusion with culture medium, an anterior chamber exchange was performed with a solution of CF (10 mg/mL, pH 5.8; Sigma-Aldrich) that had been sonicated for 15 minutes.[8–10] Eyes were then perfused with CF at 15 mm Hg for 0.5, 2, or 4 hours. At the end of the perfusion, the anterior segment was dissected and divided into quadrants. Wedges of the limbal region were removed from each quadrant. Some were immediately fixed in 3% glutaraldehyde in 0.2 M cacodylate buffer and designated "fixed" tissue. Adjacent wedges were immersed in a bath of CF (identical with perfusate solution) at 37°C for 1, 2, or 4 hours. Bath tissue at 1 hour was not shaken, whereas bath tissue at 2 and 4 hours was shaken, to prevent settling. Wedges were then fixed and designated bath tissue. Two blocks, one fixed and one bath tissue, from each of two quadrants 180° apart were processed, sectioned, and examined in a transmission electron microscope (model 1200; JEOL Peabody, MA).

RESULTS

Variations in Appearance of CF Labeling

The appearance of CF varied from single particles (12 nm) to clusters ranging in size from 76 to 486 nm. Single particles were visible in the sheath material of the connecting fibril tendons and sometimes within PEX clumps (Figs. 1, 2). Small clusters of CF could be found in the juxtacanalicular (JCT) region, on the surface of canal cells and trabecular cells, and between trabecular cells and the lamellar cortex (Fig. 3). Small clusters were also visible in the basement membrane underlying the canal, giving it a Swiss cheese appearance (Fig. 1). Larger aggregates (270–486 nm) were found in the intercellular junctions of Schlemm's canal cells after longer incubation times and on the surface of canal cells. Intercellular junctions of trabecular cells also labeled with CF (Figs. 1, 3).

Cell membranes of trabecular and canal cells of both the inner and outer wall were labeled with CF. Most cells showed a time-dependent increase in CF labeling. Trabecular cell membranes had punctate clumps of CF label scattered on their surface at 30 minutes, which by 4 hours had accumulated to

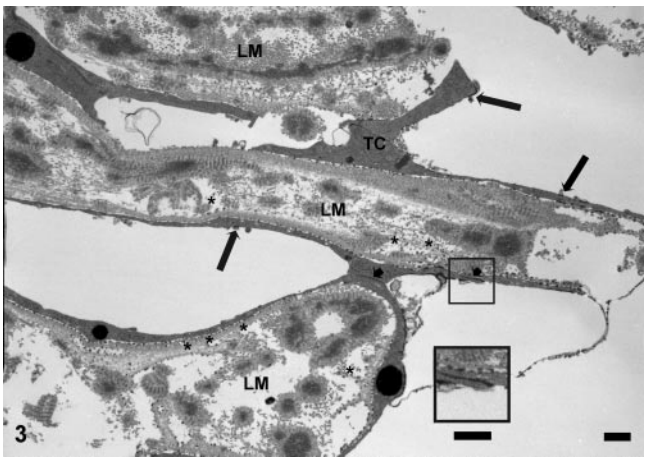

FIGURE 3. Small accumulation of CF particles on the surface of trabecular cells (TC; *arrows*), within intercellular junctions (*short arrows*), and between the trabecular cells and the lamellar cortical surface (✶, *lower left*). CF was also noted within the lamellae, near the elastic fiber core (*stars*, *center* lamellae; LM) *Inset*: CF within intercellular junction of trabecular cells. Scale bars, 0.5 μm.

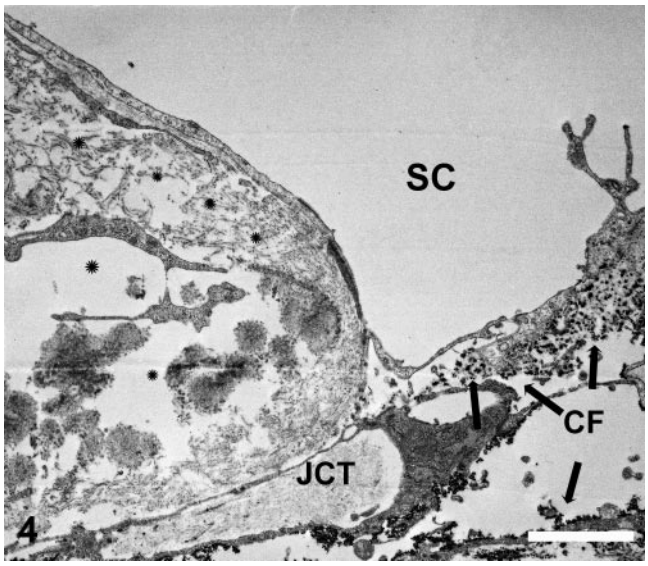

FIGURE 4. Juxtacanalicular region (JCT) of PEX eye perfused for 2 hours with CF. Segmental label was present, with a heavily labeled region (*arrows*) adjacent to region of loosely arranged PEX fibrils with no CF label (✷). Scale bar, 1.5 μm.

form multiple layers of CF coating their surface. SC cell membranes had some regions with a homogenous coating of CF, whereas other regions had clumps of CF interspersed with unlabeled regions. CF labeling of inner membranes of giant vacuoles varied from no label to small discrete clusters (Fig. 1), in contrast to the large aggregates of CF on the surfaces of the canal cell membranes. Some collector channel lumens also contained CF.

Both trabecular cells and canal endothelial cells had cytoplasmic pinocytotic vesicles containing CF. From observations of numerous cells at the 30-minute incubation, it appeared that trabecular cells actively moved CF from their outer cell surface to the basal cell surface on the lamellae. Clumps of CF were found between the trabecular cell and surface of the lamellar cortex and in the central core region of the lamellae (Fig. 3). CF was found in lamellar cores, even in areas where the overlying cell was intact or not separated from the lamellae, suggesting that transcellular movement of CF occurred. Trabecular lamellae became more heavily labeled with time.

PEX material was labeled in the peripheral portion of a clump with small clusters of CF. The interior of the clump was usually unlabeled, although label with single CF granules could be found in some clumps (Figs. 2, 4). This lack of label in the center of a PEX clump suggested that the clump was less permeable to fluid flow, similar to absence of label in the center of the elastic tendon, which is also presumed to be impermeable to fluid flow.[13]

CF Labeling with Increasing Time: Fixed Tissues

In eyes perfused with CF for 30 minutes, most labeling was in uveal and adjacent corneoscleral regions. Cell membranes in these areas were heavily coated with CF. It was also present in the intercellular junctions of the trabecular cells in these regions. Only small amounts of CF reached Schlemm's canal at 30 minutes and appeared as scattered, isolated particles in the JCT and within the anterior and midportions of the canal. Whereas CF was present in the posterior region of the canal on the luminal surface, it was not always in the JCT region underlying these areas and thus probably had reached this region of the canal via flow through the lumen. CF on the outer wall of Schlemm's canal was confined to isolated clumps of CF on the luminal surface of the endothelial cells. In one section adjacent to a collector channel, moderately heavy label of the JCT and SC endothelial surfaces of the inner wall and outer wall was present.

In eyes perfused for 2 hours, heavy label remained in the uveal and corneoscleral meshwork, and labeling was increased in the JCT regions. Segmental labeling was noted, with regions of moderately heavy label in the basement membrane material of the JCT adjacent to regions with no label (Fig. 4). Larger amounts were visible near the inner wall than the outer wall. The cytoplasmic membranes of the lumen of both the inner and outer wall canal cells were intermittently labeled, with clumps and bare regions. Although the segmental labeling was present predominately in the JCT region, isolated regions of the corneoscleral meshwork also demonstrated segmental labeling. Overall, we estimate that approximately two thirds of the total area of the TM labeled with CF. The amount of segmental labeling varied among eyes, but all demonstrated some segmental labeling.

After 4 hours of perfusion, CF staining was present throughout the meshwork, with little segmental labeling. Isolated regions without CF label were only infrequently seen in the JCT and outer corneoscleral region. Whereas most areas of the basement membrane of the canal contained CF, some regions in the basement membrane were only lightly labeled, or were not labeled at all (Fig. 5). The amount of CF in the canal and on the luminal surface of the canal cells was increased, compared with that at the shorter perfusion times, as was the amount in the JCT region of the outer wall.

PEX eyes also displayed a time-dependent increase in labeling and showed regions of segmental labeling in the meshwork. When PEX material was present adjacent to the canal, clumps of CF material were found in the peripheral matrix of the PEX material (Figs. 2, 4). Although CF could be present within clumps of PEX, it was not present in each clump. Labeling of all the interior spaces of the PEX material was never observed.

CF Labeling in Bath Tissues

In CF bath specimens, it was expected that open intertrabecular spaces would allow CF to penetrate those regions, but CF

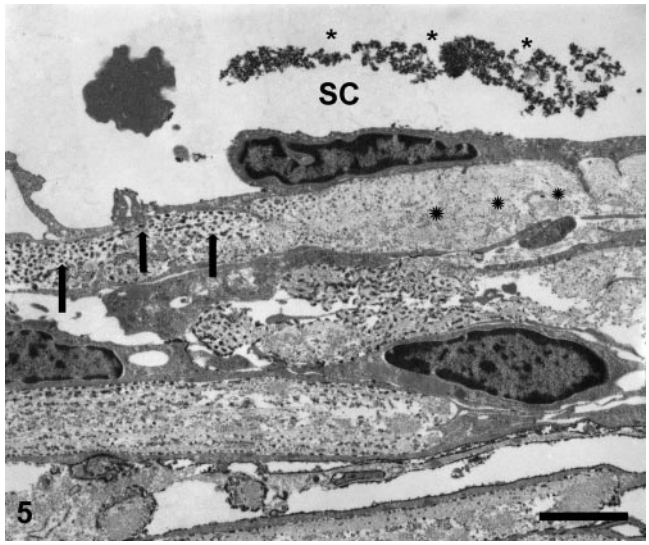

FIGURE 5. Juxtacanalicular region (JCT) of eye perfused for 4 hours with CF. Segmental label was present, with labeled region (*arrows*) adjacent to area showing light to no label (✷). CF was noted in the lumen of Schlemm's canal (✶; SC). Scale bar, 1.5 μm.

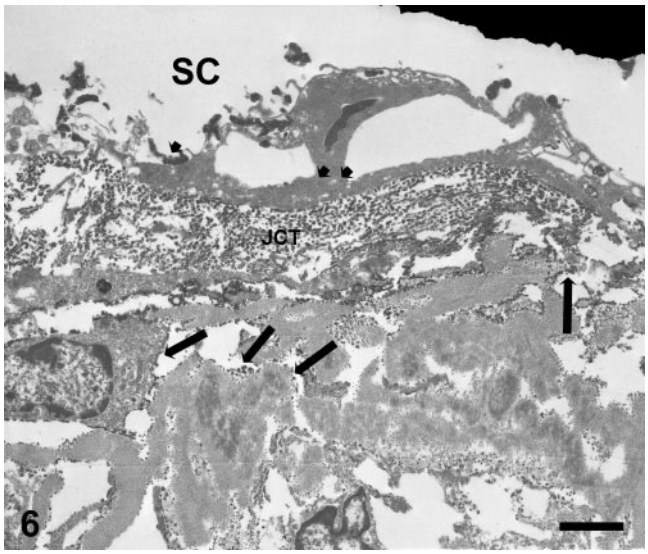

FIGURE 6. Juxtacanalicular region (JCT) of bath eye immersed in CF 4 hours. The JCT region was heavily labeled with CF. Label was also present on the surface of and within the cytoplasm of Schlemm's canal (SC) cells (*short arrows*), and outlining the sheath and tendons (*arrows*) and JCT cells. Scale bar, 2.0 μm.

did not penetrate solid areas of ECM. Thus, the periphery, but not the center, of a solid region labeled; and, when sliced open during sectioning, the inner portion of the solid region was not expected to have CF label.

CF labeling in bath tissues at 1 hour was similar to non–bath tissue, with labeling confined to the uveal and inner corneoscleral regions where it outlined the trabecular cells and beams. Similar to the findings in the non–bath tissue at 30 minutes, isolated clumps of CF were noted in Schlemm's canal. These similarities to non–bath tissue suggested that tissue incubated in a CF bath for 1 hour without shaking did not have sufficient mixing to allow CF penetration throughout the meshwork. Hence, bath time was increased, the CF bath was placed on a shaker, and the tissue wedges were trimmed smaller (1 mm width) for the remaining specimens.

Tissue wedges incubated in the CF bath for 2 hours had more labeling of the JCT and basement membrane of SC when compared with non–bath tissue. Large aggregates of CF were apparent on the luminal surface of the canal cells of the inner and outer wall. Beam cortex and cores contained CF. Some regions of the inner corneoscleral and JCT remained unlabeled. Schlemm's canal endothelial cells were labeled on the luminal surface. In comparison to the 2-hour perfusion-only tissue, CF bath tissue had more pronounced labeling of the outer wall endothelium and JCT region. It should be remembered that these CF bath specimens were from eyes first perfused with CF for 2 hours before dissection and placement into the bath. These CF bath specimens thus had a total of 4 hours' exposure to CF. This longer time than the 2-hour CF perfusion-only tissue is in keeping with the objective of this portion of the study: to determine whether nonlabeled regions would become labeled if directly exposed to CF. When compared with CF perfusion-only tissue for an equivalent length of time (4 hours), these 2-hour bath specimens had amounts of label similar to that of the 4-hour perfusion-only labeling.

Tissue in the CF bath for 4 hours had labeling throughout all areas of the meshwork, with most regions showing heavy label (Fig. 6). All regions under the inner and outer walls contained large amounts of CF, as did the lumen of the canal and also the intertrabecular spaces. CF was toxic to meshwork cells after this prolonged exposure time (8 hours total: 4 hours during perfusion, and an additional 4 hours during the CF bath), probably because of the low pH of the CF solution (pH 5.8). Nuclei were swollen and cells were fragmented or even missing. When compared with the CF perfusion-only tissues, the CF bath tissue showed heavier amounts of label, but were similar in the overall number of areas labeled.

CF Pathways

CF penetrated loose ECM material in the JCT region but was excluded from the center of dense accumulations of it in CF-perfusion specimens. Not all loose ECM was labeled, however, and we generally could not predict which regions might be labeled and which would not. Regions with CF label did not appear to have less ECM than nonlabeled regions. This suggests that fluid pathways in the JCT region are not solely determined by the extracellular material. It also suggests that extracellular material was not lost, or "washed out," of this region. Had ECM washout occurred, CF would have been carried into this region by the increased fluid flow causing the washout, and label would have been prominent in the ECM bordering these empty regions.

Two preferential flow pathways were evident. Regions of the inner wall with breaks in the endothelial lining often, but not always, had clumps of CF in the cell breaks and spilling into the canal. A second probable flow pathway was discovered in one eye perfused for 30 minutes with CF. Near the canal, CF was found in a region of loose JCT cells adjacent to the posterior portion of Schlemm's canal (Figs. 7A, 7C) but not in the JCT underlying the single lumen of the main canal (Fig. 7B). These loose cells appeared to be a distended region of the JCT that had expanded into the lumen of the canal, filling that area of the canal with elongated, thin, interconnected cell processes. Of interest, the lumen of a collector channel was present in this specimen, although it did not connect with the canal in the sections examined. CF had not yet entered any JCT regions underlying the adjacent midcanal or anterior canal lumen, despite the presence of numerous optically empty spaces in the JCT. Because this eye had been perfused only 30 minutes with CF, the distended JCT region with label was apparently a localized higher-flow region.

DISCUSSION

With increasing perfusion time with CF, more areas of the TM became labeled. This, coupled with the finding of labeling in most regions of bath-immersed tissue, suggests that the nonlabeled regions did not differ in the characteristics of the cells or ECM, but rather that CF did not reach these areas. We conclude that the segmental labeling reported by others,[8–11] and confirmed in this study, is due to preferential fluid flow pathways in the TM.

Preferential flow regions may be created by several factors: the size and the relationship of the aqueous channels, their interconnections, variations in the permeability of the ECM adjacent to Schlemm's canal, ease of fluid passage through the canal endothelial barrier, and downstream resistance, such as proximity to collector channels.[1] Other biological systems with high variability also show preferential pathways for fluid flow, such as the capillary network in the lung.[14] The diffuse label of the uveal and inner corneoscleral regions but sparse label of the JCT at 30 minutes suggests a slow, simultaneous advancement of fluid throughout the meshwork and also localized higher speed regions (accounting for the presence of the CF label in only some regions of JCT). The nonlabeled JCT regions suggest that CF had insufficient time to reach them in bulk. Even at 4 hours, a few small areas of the JCT and canal

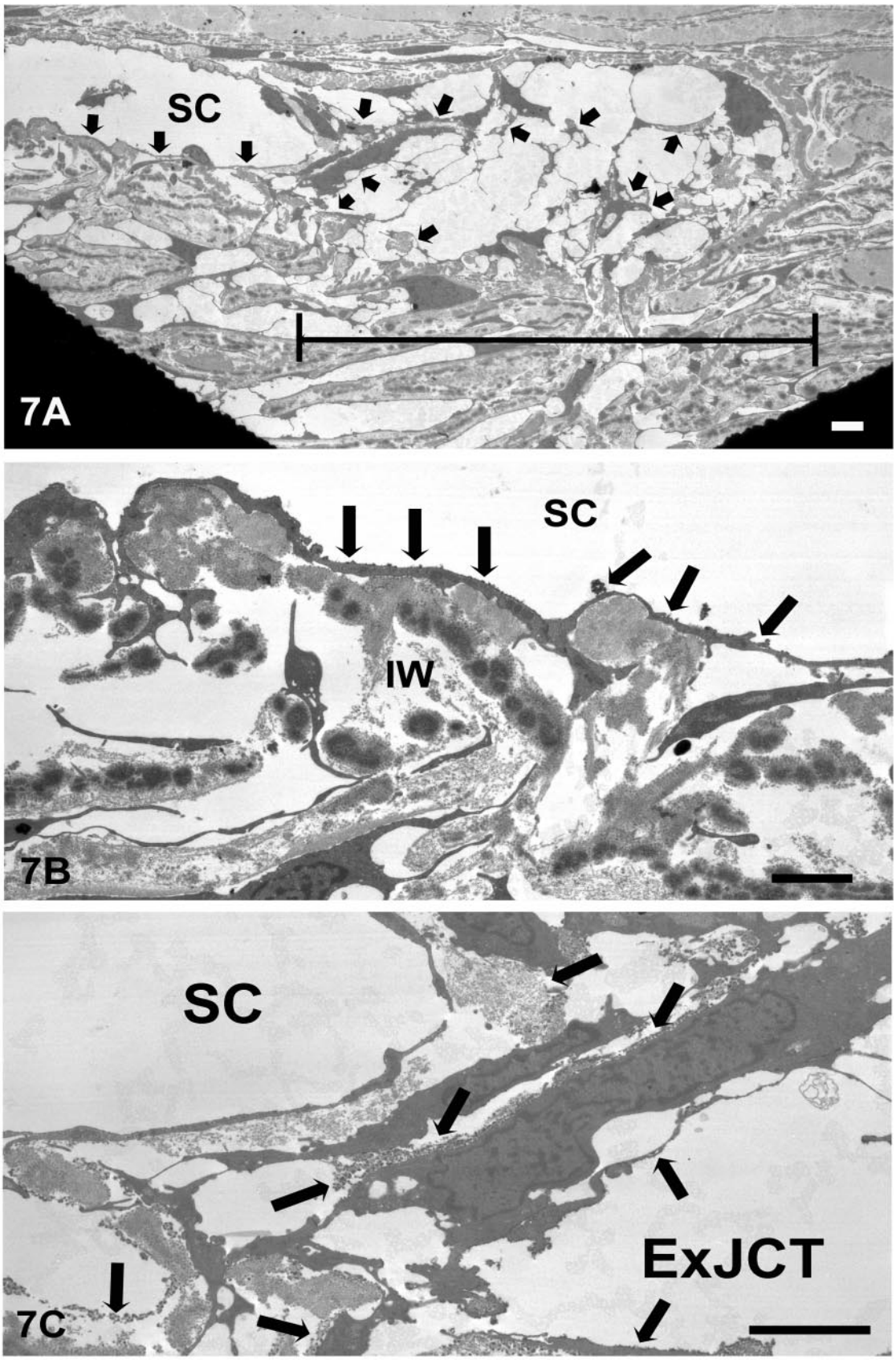

FIGURE 7. (**A**) Overview of posterior canal region, showing single lumen of Schlemm's canal (SC) and distended JCT region to the *right* of the lumen (between *bars*). CF 30-minute perfusion specimen. (**B**) Enlargement of the single-lumen region, showing CF in the canal lumen (*arrows*) but not in the JCT region. (**C**) Enlargement of expanded JCT region (ExJCT), showing CF throughout (*arrows*). Scale bars: (**A**) 5 μm; (**B**) 2.0 μm; (**C**) 2.5 μm.

basement membrane remained unlabeled, indicating some areas are "backwaters" and are not easily reached by fluid flow.

Morphologically, unlabeled regions generally appeared no different from labeled regions. We could not predict which regions might be labeled based on their ultrastructural characteristics. DeKater et al.[9] also commented that they "could not relate the pattern of tracer distribution in the different trabecular regions to pathologic changes of the TM" in glaucomatous eyes. Washout or loss of extracellular material was not evident in labeled regions (high-flow regions). One configuration appeared to relate to preferential CF label, however: a region of distended JCT that expanded into the posterior region of the canal. Label was abundant in this region, but absent from the JCT elsewhere, including the portion underlying a large single-lumen portion of the canal (Fig. 7B). We speculate that the absence of basement membrane and ECM in the distended JCT region caused less outflow resistance in this isolated area, perhaps by eliminating the "funneling" interaction of cells and extracellular material.[15]

Studies of CF have used perfusion times ranging from 20 to 90 minutes.[8–11] Investigators in these studies have used perfusion fixation and some CF labeling undoubtedly continued ahead of the front of the perfused fixative, extending the labeling time in these studies to some unknown point. To capture definite time points, we used immersion fixation. By extending our labeling times up to 4 hours, we were able to demonstrate an increase of the number of regions labeled over time. This indicates the labeled regions in shorter CF perfusions are areas in which fluid preferred to go first. The finding of more complete and diffuse labeling in bath-immersed tissues suggests the regions throughout the meshwork are similar in surface charge and binding characteristics. Although rare, nonlabeled regions in bath tissue indicate nonpenetration of the CF. These regions were probably isolated "chambers" or dead-end regions inaccessible to the CF bathing solution, and thus never received label. After the tissue wedges were sectioned, these regions appeared open and connected with adjacent regions. The three-dimensional flow pathways were lost in the two-dimensional sections, giving the puzzling finding of adjacent labeled and nonlabeled regions in the bath tissue.

Our segmental labeling results with CF agreed with the study by Ethier and Chan,[10] who also found segmental label in normal eyes. deKater et al.[9] found homogeneous label in normal human eyes, using a different perfusion technique. In that study, CF was perfused immediately into the eye while the eye was at a low intraocular pressure, whereas in our study and that of Ethier and Chan,[10] eyes were first perfused with non-CF fluid for 1 hour, to reinflate the eyes and establish normal outflow conditions before adding the CF.[1,10] It is likely that an initial perfusion with CF results in greater amounts entering the eye, as the eye becomes inflated and pressurized, and thus label appears in more locations.

The presence of CF in the trabecular lamellae indicates these structures are continually bathed in fluid. Trabecular cells appeared actively to move CF from their outer cell surface to the lamellae. Melamed et al.[8] felt the CF gained access to the lamellar cores through detached trabecular cells or through intercellular separations. In contrast to this, we found CF in lamellar cores, even in areas where the overlying cell was intact or not separated from the lamellae, suggesting that transcellular movement of CF occurred.

The absence of CF from the center of most clumps of PEX material suggests it is not freely permeable. PEX clumps thus could act like boulders in a stream, obstructing some regions and effectively decreasing the total area of outflow pathways. PEX material was often labeled in the periphery of clumps, but also sometimes in the center of clumps, with single particles of CF. We could not predict which PEX clumps would have label. The density of the clump did not always correlate with labeling (compare Figs. 2 and 4). PEX consists of elastic material (fibrillin, tropoelastin, α-elastin, vitronectin, and microfibril-associated glycoprotein [MAGP])[16] and also contains basement membrane proteins (heparan sulfate proteoglycan, enactin, and carbohydrate moieties).[17] Variations in the basement membrane components could account for relative difference in PEX permeability and labeling, especially as most of these components are lost in processing.

Preferential fluid flow pathways are present in the living eye, as demonstrated by the segmental pigmentation of the TM often observed with gonioscopy. Preferential flow regions may be more common in the enucleated eye, however, due to the loss of ciliary muscle tone. Some ciliary muscle tendons connect with the meshwork and help keep the lamellae separated.[18–20] Active contraction and relaxation of the ciliary muscle during life causes the meshwork and canal to expand or contract, altering the shape and relationship of the aqueous channels in the intertrabecular spaces and also in the JCT.[18–20] This may increase the number of "active flow" channels and result in fewer regions of preferential flow than observed in the enucleated eye. Such opening of additional aqueous channels is part of the facility-increasing mechanism of pilocarpine.[18–20] It is tempting to speculate that the loss of accommodative ability with age, in part due to decreased contraction of the ciliary muscle, could lead to less interconnections of aqueous pathways through the meshwork, and increase preferential flow pathways at the expense of less overall aqueous drainage routes through the meshwork. This is similar to a concept suggested by Kaufman that periodic contractions of the ciliary muscle may prevent "silting in," or the buildup of extracellular material, of the meshwork channels.[21] Our CF findings confirm the concept that preferential flow pathways exist in human TM.

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E R R A T A

Errata in: "Fundus Autofluorescence and Fundus Perimetry in the Junctional Zone of Geographic Atrophy in Patients with Age-Related Macular Degeneration" by Schmitz-Valckenberg et al. (*Invest Ophthalmol Vis Sci.* 2004;45:4470–4476).

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Footnote 2 should read: [2]Contributed equally to the work and therefore should be considered equivalent authors.