Aqueous Humor Stimulates the Migration of Human Trabecular Meshwork Cells In Vitro

Penny Hogg, Mary Calthorpe, Mark Batterbury, and Ian Grierson

PURPOSE. Depletion of trabecular meshwork cell numbers is a feature of the outflow system in aging and in primary open-angle glaucoma. It is possible that migration stimulated by factors present in aqueous humor may contribute to the cell loss. This investigation assessed the chemoattractant potential of glaucomatous and nonglaucomatous human aqueous humor and fibronectin, one of its constituents, on a range of cultured trabecular meshwork cell lines.

METHODS. Migration was assessed in 48-well modified Boyden chambers. The potential migratory stimulants were soluble fibronectin and glaucomatous and nonglaucomatous aqueous humor. The glaucomatous aqueous samples were collected from patients undergoing trabeculotomy for primary open-angle glaucoma and the normal aqueous from normal bovine eyes and patients undergoing cataract surgery. The target cell types were normal human and bovine meshwork cells grown from explants and two human transformed meshwork cell lines from a normal (HTM-5) and a glaucomatous (HTM-3) source.

RESULTS. Soluble fibronectin stimulated all the target cells to migrate with an optimal concentration ranging from 1 to 30 μg/ml, and Zigmond Hirsch checkerboard analysis indicated that both chemotaxis and chemokinesis took place. All the aqueous humor samples stimulated migration of the meshwork cell lines at an optimal concentration of 200 μl/ml. Glaucomatous aqueous humor stimulated a greater migratory response than nonglaucomatous aqueous for two of the four target cell types ($P \leq 0.03$). Neutralization of the fibronectin content of nonglaucomatous and glaucomatous aqueous by addition of excess anti-fibronectin antibody indicated that fibronectin could account for 35% to 80% of the migratory activity of the aqueous.

CONCLUSIONS. Aqueous humor contains potentially powerful chemoattractants for trabecular meshwork cells. The activity of one of these constituents, fibronectin, has been accounted for by this study. Glaucomatous aqueous appears to be as good and in some cases a better migratory stimulant than nonglaucomatous aqueous in vitro. The migratory evidence points to a trend that may help to explain cell loss in the aging meshwork and possibly some of the extra loss in primary open-angle glaucoma. (*Invest Ophthalmol Vis Sci.* 2000;41:1091–1098)

The cell population of the outflow system of the normal eye reduces with increasing age,[1-4] and this cell loss may precipitate some of the structural alterations that occur during the aging process such as trabecular thickening and trabecular fusion.[3] The age-related cell loss is even more pronounced in patients with primary open-angle glaucoma (POAG) than in age-matched normals,[2-5] and it has been suggested that excessive cell loss is an early, and perhaps the primary, pathologic event in the outflow system in POAG.[2,5,6] The mechanism of cell loss and the environmental factors contributing to it are not known. The meshwork cell loss may

be brought about by cell death caused by mechanical stress[7] or by noxious insult such as free radical attack.[8,9]

We have suggested a further mechanism for meshwork cell depletion in health and disease,[4,5,10] namely detachment from the trabeculae and migration from the outflow system. Meshwork cells, provoked by a variety of stimuli, become "activated," detach from their neighboring cells on the trabeculae, undergo shape changes and then migrate to Schlemm's canal and pass through the endothelium into the lumen of the canal.[11] The "activation" process is associated with excessive phagocytosis, inflammation, injury, or a combination of all three processes.[11-14] It is probable in these circumstances that the aqueous fluid, which bathes the meshwork cells, contains motogenic factors that stimulate meshwork cell migration. It is also possible that normal aqueous humor and the aqueous humor from POAG patients contain motogens. If so, then the steady attrition of meshwork cells might be caused by a slow version of the process seen after inflammatory and particulate insult.[5,11-14] Electron microscopic studies show meshwork cells partially detached from the trabeculae in the normal aging meshwork[3] and in trabeculectomy specimens from POAGs.[7]

Migration is likely to be a difficult event to study in vivo because cell loss has been calculated to be at most only in the

region of 20 cells per day.[4] In addition, any chemoattractants found in aqueous humor are likely to be there in very small quantities. The microchemoattraction chamber assay is a sensitive procedure that serves both to quantify and to analyze migration in vitro. In the case of meshwork cell migration it also acts to amplify in vitro the small migratory changes that may be stimulated by aqueous fluid in vivo. Our group has previously used chemoattraction chambers to study whether bovine meshwork cells are migratory, and we have shown that bovine cells are responsive to various stimuli.[5,15–17] These include conditioned media collected from cultured corneal endothelium and scleral fibroblasts,[16] the glycoproteins fibronectin (Fn)[5,15] and laminin.[17] In addition, platelet-derived growth factor is a highly potent migratory stimulant, whereas EGF and bFGF show no activity at all; others lie somewhere in between.[17] When bovine aqueous was used as a stimulant the bovine meshwork cells responded to it as well as they did to optimal concentrations of soluble fibronectin (sFn).[17] As yet, however, our studies have not looked at whether human, and in particular POAG, aqueous humor acts as a migratory stimulus for cultured human meshwork cells.

Therefore, this investigation aims to establish whether or not aqueous humor is a chemoattractant for a range of human trabecular meshwork cell lines and also if glaucomatous aqueous specimens from POAG patients elicit a greater migratory response than nonglaucomatous aqueous (from routine cataract patients). In addition, this study aims to determine whether sFn, one the major components of the aqueous,[18,19] stimulates human meshwork cells to migrate and contributes to the attraction of aqueous. Our hypothesis is that if the meshwork cell migration caused by chemoattractants in aqueous explains part of the cell loss in aging, then it follows that the excessive cell loss associated with POAG may be due to glaucomatous aqueous being a more effective chemoattractant than nonglaucomatous aqueous.

METHODS

Tissue Culture

The trabecular meshwork from six human eyes was dissected using standard procedures,[20] and they were used as explants in 25-cm^2 tissue culture flasks (explants 14, 15, 19, 20, 22, and 59). The eyes came from donors (between 12 and 48 hours postmortem; age range, 5–38 years) whose corneas had been removed for grafting. The explants were incubated for 2 weeks at 37°C in 5% CO$_2$, in Ham's F10 Nutrient Media (F10) culture medium, containing 20% fetal calf serum (FCS) supplemented with L-glutamine (2 mM), penicillin (100 U/ml) with streptomycin (100 μg/ml) solution, Fungizone (amphotericin B 2.5 μg/ml), and 1.2 mg/ml sodium bicarbonate (all from Life Technologies, Paisley, UK). When cells were observed growing out of the explant the cultures were then fed with F10 and 10% FCS twice a week, and the explant was removed when a healthy monolayer was established. At confluence the meshwork cells were trypsinized (0.25% trypsin and 0.02% EDTA) and passaged at a split ratio of not more than 1:4, and cultures between 3rd and 5th passage were used in our experiments. The cell lines were used separately for the subsequent experiments. The phenotype of our cells conformed to those previously described in the literature for both human[21] and bovine[22,23] cultured meshwork cells.

Additional meshwork cells were provided by two transformed cell lines, HTM-5 and HTM-3 (a gift from Abbot Clark, Alcon Laboratories, Fort Worth, TX). The transformation characteristics and procedures have been reported previously.[24] Preconfluent cultures were infected with 25 plaque-forming units/cell of SV40.[25] Transformed cells came from two sources, an 18-year-old nonglaucomatous man (HTM-5) and a 72-year-old man who had POAG controlled by β-blockers at the time of death (HTM-3). The cells were fed routinely with 10% FCS in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine (Life Technologies), and the cells were subcultured at a split level of 1:5.

Wild-type bovine meshwork cells [BTM(w)] served as a standard for comparison with human cells and were established in culture using the procedure first reported by our group.[22] Throughout the investigation the bovine cells were passaged at a split level of 1:5, and as for the wild-type human cells the bovine cultures were used between the 3rd and 5th passages.

Chemoattractants

Bovine aqueous humor was taken within 4 hours postmortem from eyes collected from the abattoir and placed on ice. Between 600 and 750 μl was removed from each eye through a 25-gauge needle attached to a 1-ml syringe. Care was taken to avoid iris contact. A total of 12 specimens of bovine aqueous were used for the study. Human aqueous humor was available from two sources, cataract patients (nonglaucomatous) and patients undergoing trabeculectomy for POAG (glaucomatous). All specimens were taken as the first intraocular maneuver as close to the start of surgery as possible. The fluid was removed with a 1-ml syringe attached to a 27-gauge needle. Iris, lens, and corneal endothelial touch was avoided at all times, and a volume of approximately 100 μl was obtained from each patient. A total of 81 specimens of aqueous were taken from eyes with cataracts and 63 from glaucomatous eyes whose donors had undergone maximal medical therapy. The percentage of the glaucomatous specimens used in which the patients also had cataracts was approximately 5% (3 from a total of 63 POAG specimens). The age range for the cataract donors was 52 to 100 years (mean age $\pm$ SD, 73.6 $\pm$ 9.4 years) and 30 to 86 years for the POAG donors (mean age $\pm$ SD, 62.2 $\pm$ 13.8 years). After removal the specimens were immediately transferred to siliconized tubes, immersed in liquid nitrogen, and then stored at -80°C. Storage for up to 1 year at this temperature was shown by us to have little adverse effect on the migratory activity in the human or bovine fluids. For the migration studies the aqueous was diluted to concentrations ranging from 100 to 1000 μl/ml in serum-free medium.

Soluble fibronectin from human and bovine sources was used (Sigma, Poole, UK). Both types of sFn were checked for purity by gel electrophoresis. Contaminants were at the limit of resolution with Coomassie blue and were thought to be of such low levels as to be inconsequential.

Antibody Neutralization

The contribution of sFn to the chemoattractant properties of glaucomatous and nonglaucomatous aqueous was estimated by antibody neutralization experiments. A polyclonal antibody (rabbit anti-human fibronectin from Dako, High Wycombe, UK) was used to optimize blocking of the active binding sites

present on fibronectin. The human aqueous samples were pooled for this part of the study.

The dilution of human anti-sFn antibody needed to block the activity of sFn in aqueous samples had been identified in our previous studies on bovine aqueous[17] by the Aucterlony immunodiffusion method. The antibody needed to be five times the concentration of sFn for complete equivalence. In addition the antibody was used in a series of dilutions in serum-free medium (1/10,000, 1/1000, 1/500, and 1/100) to find the concentration that blocked activity due to sFn. Based on these findings the antibody was added in excess of the maximum expected concentration of sFn in our samples of human aqueous (5.8 μg/ml). Previous analysis by enzyme-linked immunosorbent assay of 48 of the specimens of aqueous (cataract aqueous, $n = 29$; POAG aqueous, $n = 19$) used in the migration studies had produced an sFn content that ranged from 0.7 to 5.8 μg/ml with a mean $\pm$ SD of 1.2 ± 0.6 μg/ml for the cataract and 1.9 ± 1.3 μg/ml for the POAG specimens (Khaw P, personal communication, September 1992).

Migration

Migration assays were conducted in 48-well microchemoattraction chambers (Neuroprobe, Cabin John, MD) using a method we described previously.[15] The chemoattractants were placed in the lower wells of the chamber and covered by an upper set of wells separated by a gelatin–coated[15] polycarbonate membrane with pores of 10 μm diameter. The cells were prepared for the migration chamber as has been described in our bovine studies.[15,17] They were placed in each of the upper wells at a concentration of 40,000 cells per well, and the chambers were incubated at 37°C in 5% CO_2 for 4 hours after which the membrane was removed and fixed in ethanol for 15 seconds. The cells on the membranes were stained with hematoxylin, washed in distilled water, and mounted. Cells that had migrated through the pores could be visualized on the lower surface of the membrane and were counted under the $\times 100$ oil-immersion objective of a light microscope (Nikon Optiphot) in 20 fields per well and four wells per concentration.

Statistical analysis of data was performed using Unistat Statistical Package (version 4.5.01; Unistat, London, UK). An unpaired t-test was used to compare single groups or a nonparametric test (Mann–Whitney) in instances of nonnormal distribution of data. To test for significant differences between more than two groups, ANOVA with a Bonferroni-type correction (Duncan's multiple range test) was used.

RESULTS

Soluble Fibronectin

The target cells were stimulated to migrate by bovine sFn in a dose-dependent manner. The HTM(w) [cell lines HTM(w) 14, 15, and 19] showed an optimal migratory response at 1 to 5 μg/ml sFn (Fig. 1A) and the HTM-5 and HTM-3 at 20 μg/ml sFn (Fig. 1B). The BTM(w) cells were used as the positive control, and previous studies[17] had shown their optimal concentration for migration to bovine sFn to be 30 μg/ml (Fig. 1B). Zigmond Hirsch checkerboard analysis indicated that the migratory response of the human meshwork cell lines HTM(w),[22] HTM-5 and HTM-3 (Fig. 2), to sFn was both chemokinetic and chemotactic. The optimal concentration of bovine sFn for each cell

type was adopted as the positive control in subsequent experiments.

Migration of the human transformed cell lines HTM-5 and HTM-3 to human sFn was also dose dependent in the range 0.5 to 60 μg/ml and the optimal migratory response of the cells was at a concentration of 20 μg/ml sFn (Fig. 3). This concentration of human sFn was subsequently used as the positive control in the human anti-sFn antibody neutralization studies.

Aqueous Humor

All the target meshwork cell lines were stimulated to migrate positively toward bovine aqueous humor, although the human cell lines were less effective than the bovine meshwork cells (Fig. 4). The response of each of the cell lines was significantly higher than serum-free medium, which served as a negative control (unpaired t-test; $P \leq 0.002$). A standardized concentration of 200 μl/ml (20%) aqueous was used throughout these investigations because it produced the optimum migration during preliminary dose–response runs (data not shown). Concentrations of 500 μl/ml and above were either no better at stimulating meshwork cell migration or they showed a drop in effectiveness.

Human aqueous humor samples from cataract and POAG patients also were effective stimulants for the migration of all the test meshwork cell types (Fig. 5). The human and bovine cells were tested against a large series of aqueous humor samples [HTM(w) cataract, $n = 23$, POAG, $n = 13$; HTM-5 and HTM-3 cataract, $n = 25$, POAG, $n = 25$; BTM(w) cataract, $n = 12$, POAG, $n = 12$]. As with bovine aqueous, human cataract and POAG aqueous both were dose-optimal stimulants at a 200 μl/ml concentration (data not shown), and the levels of migratory stimulation were highly significant when compared with serum-free medium (unpaired t-test; $P \leq 0.005$). Cataract aqueous produced its highest response from the human wild-type cells [HTM(w) 25], and they were approximately three times more effective at migration than the human transformed cell line and the bovine cells (Mann–Whitney; $P \leq 0.007$). A similar order of response was provoked by POAG aqueous with the HTM-5, HTM-3, and BTM(w) cells showing one third to one half the response of the HTM(w) cells (Mann–Whitney; $P \leq 0.009$).

It was clear that both cataract and POAG aqueous were strong chemoattractant solutions for a range of cultured meshwork cells. The question arises whether there was a substantial difference in effectiveness of the two types of aqueous. The response of all four cell systems to POAG aqueous was more pronounced than that to cataract aqueous (Fig. 5). However, with the two sets of cells, BTM(w) and HTM-3, the difference was significant (Mann–Whitney; $P < 0.0002$ and $P < 0.03$, respectively) but not for HTM-5 ($P < 0.06$) or HTM(w) ($P < 0.09$) cells.

Antibody Neutralization with Anti-sFn in Human Aqueous Humor

We wished to determine by neutralization experiments how much of the chemoattraction in aqueous humor was due to the presence of sFn. Where necessary, human aqueous samples were pooled to provide sufficient test fluid for our experimental needs. In addition, the study was restricted to only the transformed cell lines, HTM-3 and HTM-5.

To aliquots of pooled cataract aqueous, at the working dilution of 200 μl/ml, various concentrations of anti-sFn anti-
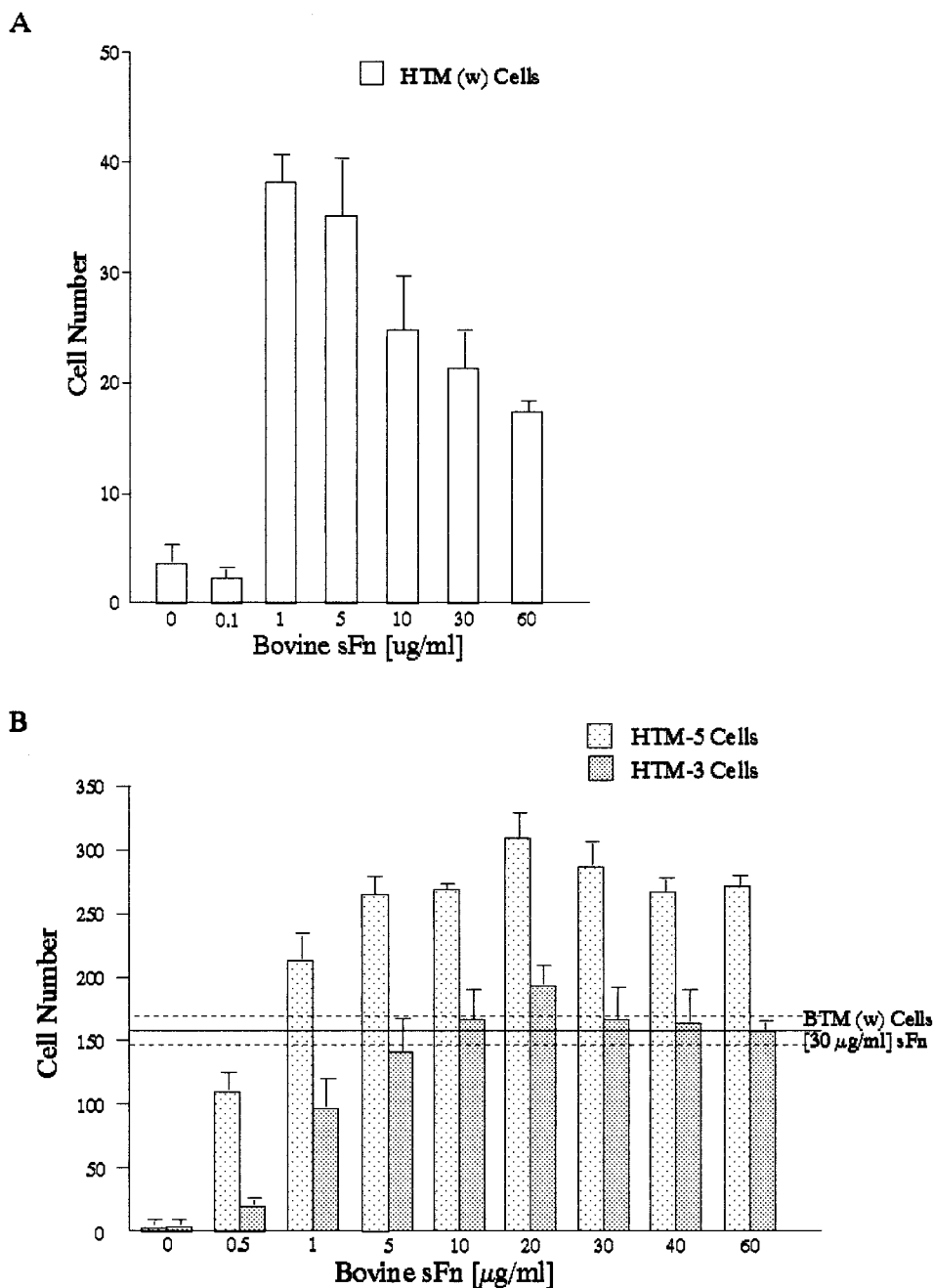
A

B

FIGURE 1. Dose–response histograms of the migratory response of cultured HTM(w) [HTM(w) 14, 15, and 19; **A**] and transformed human cell lines HTM-5 and HTM-3 to sFn (**B**). Each column represents the mean (±SEM) of at least four wells, and the experiments were repeated three times. In the histogram (**B**) the optimal migration to the bovine control cells [BTM(w)] is represented by *dotted lines*. The optimal migratory response for the HTM(w) was between 1 and 5 μg/ml sFn, and the HTM-5 and HTM-3 showed an optimal response of 20 μg/ml.

body were added. Migration decreased with increasing concentrations of antibody, showing that neutralization was taking place; the neutralization effect bottomed out between 1/100 and 1/500 of antibody in aqueous (equivalent to 100 to 20 μg/ml protein; Fig. 6A). The higher concentration of 1/100 (100 μg/ml) of antibody was adopted as our optimum neutralizing dose; and when added to the background control solution, serum-free medium had no recognizable effect on migration (unpaired *t*-test; NS; Fig. 6B). In addition, when 100 μg/ml of antibody was added to a 1.5 μg/ml solution of sFn, it completely inhibited its chemoattraction (Fig. 6B; 1.5 μg/ml was our highest estimate and 0.25 μg/ml the average for sFn content in a 20% dilution of aqueous; Khaw P, personal communication).

The migratory stimulation of pooled cataract aqueous (3 combined samples, diluted to 200 μl/ml) on meshwork cells was reduced significantly when neutralizing anti-sFn antibody was added (unpaired *t*-test; $P < 0.001$). The reduction was 64% (Fig. 7A, columns 4 and 5) in this experimental run on HTM-5 cells and 60% for the HTM-3 (data not shown), but it varied between 35% and 65% for different experimental runs. The effect of boiling the aqueous reduced migratory activity by as much as 85% (Fig. 7A, column 6). In a further series of experiments, the migration of HTM-5 cells to cataract and POAG aqueous (3 combined samples of each diluted to 200 μl/ml) was compared with and without the addition of anti-sFn antibody (Fig. 7B). The neutralization reduced the migratory activity for the cataract aqueous by 35% (column 3) and the POAG

Upper Wells

sFn [μg/ml]	0	0.5	5.0	20
0	1.8 ± 0.6	2.2 ± 0.9	1.2 ± 0.6	1.7 ± 0.3
0.5	26.2 ± 6.3	31.5 ± 6.0	30.4 ± 3.9	35.5 ± 4.8
5.0	121.7 ± 13.3	122.0 ± 13.8	117.2 ± 14.8	182.0 ± 47.2
20	161.8 ± 17.0	182.3 ± 12.0	154.5 ± 19.9	161.5 ± 10.7

Lower Wells (row label, left axis)

HTM-3 Cells

FIGURE 2. Zigmond Hirsch checkerboard analysis of the migratory response of transformed human meshwork cells HTM-3 to bovine sFn. Each column represents the mean (±SEM) of at least six wells, and the experiments were repeated three times. The chemoattractant sFn stimulated an increase in chemotactic (*vertical column*) and chemokinetic activity (*diagonal column*) for the HTM(w).

aqueous by 80% (column 5; unpaired *t*-test; $P = 0.1$ and $P < 0.005$, respectively). Generally, the percentage reduction of migratory activity produced by neutralizing antibody on cataract and POAG aqueous was variable but overlapping.
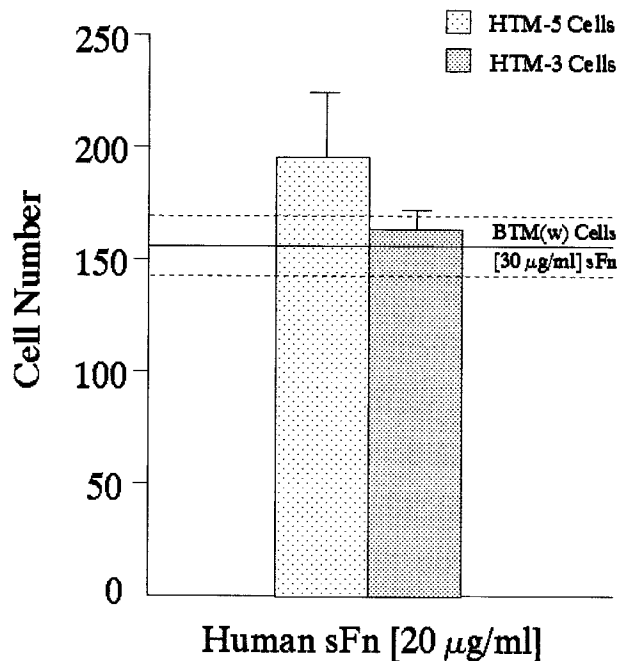
FIGURE 3. Histogram of the optimal migratory response of HTM-5 and HTM-3 cells to human sFn. The migration to the BTM(W) control cells (±SEM) is represented by *dotted lines*. Each column represents the mean (±SEM) of at least four wells, and the experiments were repeated at least four times.
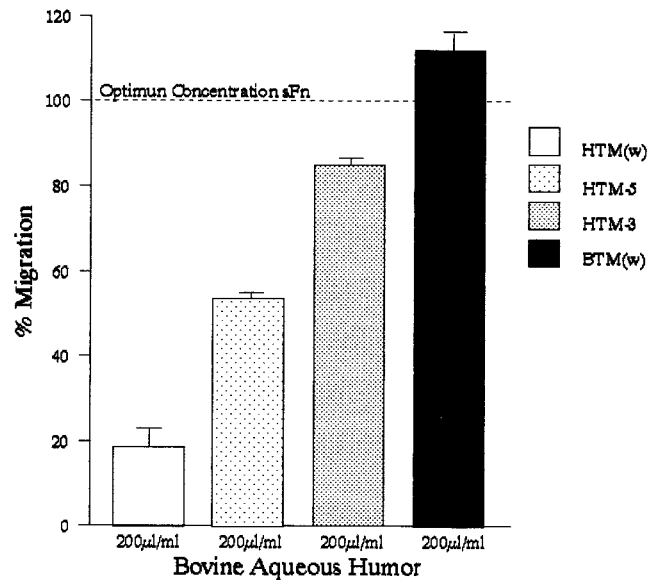
FIGURE 4. Histograms of the migratory response of HTM(w) [HTM(w) 59], HTM-5 and HTM-3, to unpooled ($n = 12$) samples of bovine aqueous humor. Migration was expressed as a percentage of each cell type's response to their optimum concentration of sFn (100%). Each column represents the percentage mean (and SEM) of at least four wells, and the experiments were repeated three times.

DISCUSSION

We have suggested that the meshwork cell loss, which is associated with aging of the outflow system,[1–4] and which is more marked in POAG,[2–5] may result in part from meshwork cells being stimulated to migrate away from the trabeculae.[4,5,10] The stimuli for migration we would propose are chemoattractants present in the aqueous humor.[17] We know that aqueous has a wide range of glycoproteins and growth factors as its constituents,[26] and these can serve not only as mitogens but also as motogens for various cell types, including meshwork cells.[15,17]

Meshwork cell migration in vivo is based only on circumstantial and qualitative evidence. For example, migratory meshwork cells have been noted after the entry of debris into the outflow system[11,13]; however, there has also been an influx of inflammatory cells to complicate identification. In noninflammatory situations such as aging, POAG, and other forms of glaucoma, where meshwork cell migration may or may not be occurring, the migratory event is likely to be too infrequent for meaningful evaluation. If migration accounted for all the meshwork cell loss in aging, for example, then the rate of attrition could be as low as 20 cells per day.[4] It is therefore not surprising that little or nothing is known about the fate of migrating meshwork cells in the outflow system or even the conditions that specifically initiate the migratory activity in the first place.

Our investigations inevitably have had to turn to the simplest possible test systems to try and dissect the complex events in vivo, particularly with respect to the stimuli that may or may not provoke meshwork cell migration. In vitro analysis of meshwork cell migration has the severe limitation of being remote from the physiological and pathologic events in vivo, so the findings need to be interpreted with caution. On the
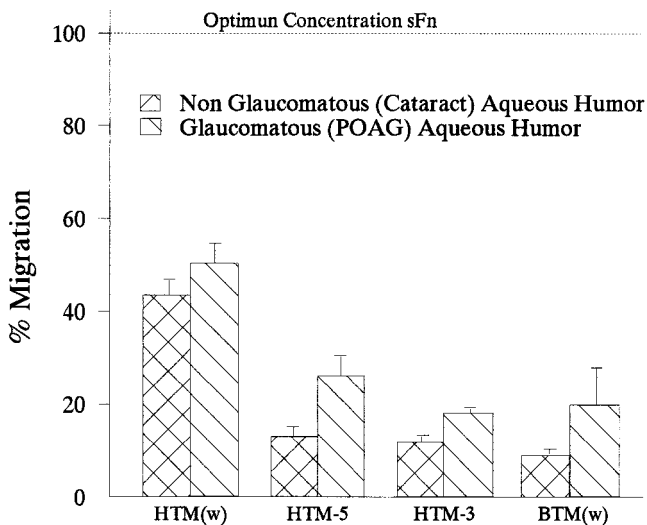
FIGURE 5. A histogram comparing the percentage migration of HTM(w) [HTM(w) 25], HTM-5, HTM-3, and BTM(w) to unpooled samples of human nonglaucomatous (cataract) and glaucomatous (POAG) human aqueous humor. The sample sizes were as follows: cataract aqueous [HTM(w), $n = 23$; HTM-5 and HTM-3, $n = 25$; and BTM(w), $n = 12$] and POAG aqueous [HTM(w), $n = 13$; HTM-5 and HTM-3, $n = 25$; and BTM(w), $n = 12$]. Migration was expressed as a percentage of each cell type's response to their optimum concentration of sFn (100%). Each column represents the mean (±SEM) of a minimum of four wells. The glaucomatous aqueous humor stimulated greater migration than nonglaucomatous aqueous from all the cell types tested. The increases in migration for the BTM(w) and HTM-3 were significant ($P < 0.0002$ and $P < 0.03$, respectively); however, the increases for HTM(w) and HTM-5 were not significant to 95% confidence limits ($P < 0.09$ and $P < 0.06$, respectively).

other hand, our 48-well modified Boyden chamber assay has particular strengths. The assay is miniaturized so that small samples can be studied, the migration can be quantified readily, the assay is reasonably reproducible, and complex environments can be modeled as a series of simple steps.

Previous investigations from our laboratory have established that bovine meshwork cells migrate positively to chemoattractants that vary from glycoproteins to growth factors some of which are known constituents of aqueous humor.[15–17] Bovine meshwork cells also migrate very effectively to their own aqueous humor.[5,17] The present study has shown, for the first time, that the situation is similar in humans. We found that bovine aqueous, aqueous from cataract patients, and aqueous from patients with POAG were powerful chemoattractants for a range of cultured human meshwork cells.

The response of our cells to human aqueous peaked at a 20% dilution in serum-free medium as was also the case with bovine aqueous humor. Why didn't 100% aqueous produce a more marked effect than our optimum dilution, given that its constituent chemoattractants would be 5 times more concentrated? A potential explanation for our result might be that the key chemoattractants were at supermaximal levels in undiluted aqueous humor. However, this was not borne out by our previous investigations of bovine meshwork cells, which showed that all the active constituents investigated were below their migratory optimum in aqueous humor.[15,16] For that matter, the present study has shown that, with sFn at least, the

situation was the same in the human test system where optimum migration was produced by 5 to 20 μg/ml, a concentration that was far higher than that normally found in aqueous.
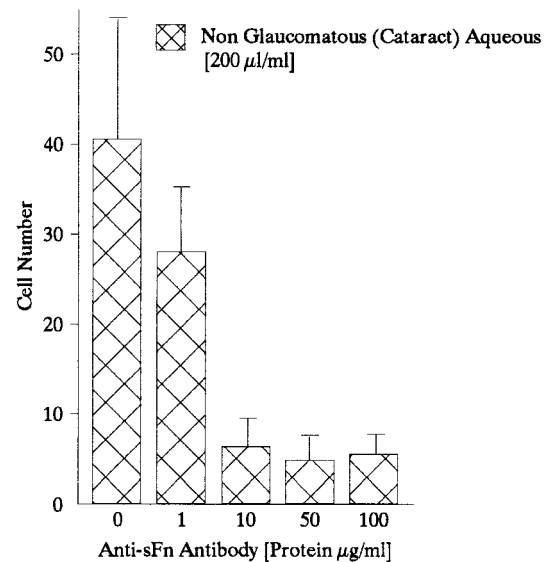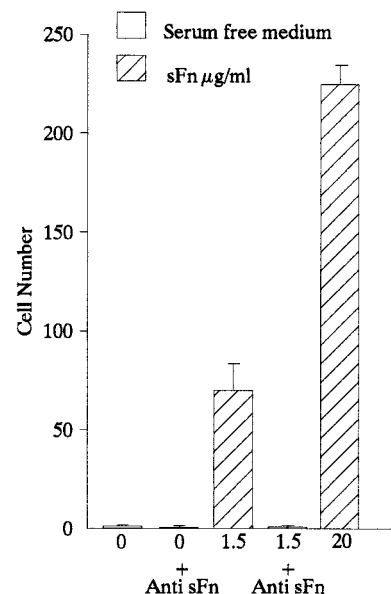

FIGURE 6. Histograms of the addition of anti-sFn polyclonal antibody to pooled ($n = 3$ per run) specimens of nonglaucomatous (cataract) aqueous. Each column represents the percentage mean (±SEM) of four wells, and the experiments were repeated twice. In histogram (**A**) increasing concentrations of antibody protein show a decrease in the migratory response of HTM-5 cells to human sFn. The response reached a plateau between 50 and 100 μg/ml of protein. Histogram (**B**) illustrates that the addition of 1/100 (100 μg/ml) anti-sFn antibody to the maximum expected concentration of sFn in 200 μl/ml (20%) of human aqueous reduced migratory activity to basal levels. Addition of 100 μg/ml antibody to the negative control of serum-free medium had no effect (NS).
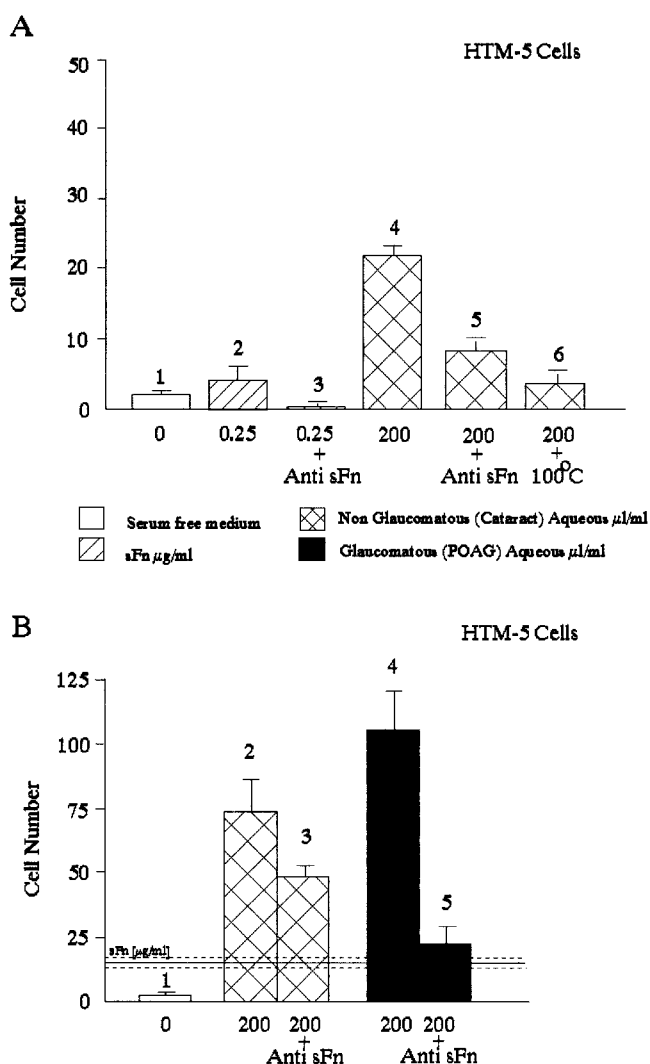
FIGURE 7. Histograms showing the extent of the migratory response of HTM-5 cells to pooled samples of cataract aqueous humor (**A**) and cataract and POAG aqueous humor (**B**) after using anti-sFn antibody neutralization and heat treatment (3 minutes at 100°C). Each column represents the mean (±SEM) of at least four wells, and the experiments were repeated at least three times. In histogram (**A**) migration to 20% of the mean level of sFn estimated in our samples ($n = 29$) of cataract aqueous (i.e., 0.25 μg/ml sFn; *column 2*) was less than the migration of HTM-5 cells to 200 μl/ml aqueous (*column 4*; $P > 0.0002$). Addition of antibody left 36% activity in comparison to 200 μl/ml aqueous (*column 5*). Boiling the aqueous for 3 minutes left 15% of activity (*column 6*). In histogram (**B**) comparison between the migration of HTM-5 to pooled cataract (*column 2*) and POAG (*column 4*) aqueous showed an increase that again was not significant ($P = 0.2$). Addition of anti-sFn antibody left 65% activity for the cataract aqueous (*column 3*) and 25% activity for the POAG aqueous (*column 5*).

Aqueous humor has a complex cocktail of constituents, and it need not be that all constituents either stimulate migration or are neutral. It may be that there are inhibitors and that this inhibition impedes the activation of migration at higher concentrations of aqueous. The variable, but potent, family of transforming growth factor-βs can be powerful mitogenic inhibitors and also are potential motogenic inhibitors.[27] Family members are present in the aqueous humor,[28] and although they have a minimal effect on migration of meshwork cells on

their own,[17] their action in the presence of known stimulants has not yet been studied.

Another possible explanation comes from the work of Burke et al.[29] They found that although 20% aqueous samples stimulated the growth of cultured ocular fibroblasts, higher concentrations were inhibitory. They explained the anomaly on the basis of poor cell survival in 100% aqueous. Our migration assays were run for a far shorter period than was needed for proliferation experiments, and we found no evidence of aqueous toxicity at high concentrations. None-the-less, our meshwork cells, adapted to culture conditions, may have found near or full strength aqueous not to have been a particularly favorable environment.

We were able to show clearly that aqueous in general was a powerful chemoattractant for human meshwork cells; however, the following question arises: Was there a difference between cataract and POAG sources? The answer was not clear-cut because with two of our cell systems there was significantly greater migration to the POAG aqueous [BTM(w) and HTM-3], but in the other two the difference, although favoring POAG aqueous, was not significant [HTM-5 and HTM(w)]. There was an overall trend that indicated that the POAG aqueous was the more powerful attractant, but it was not entirely conclusive. The question arises whether by evaluating more specimens a more definitive answer would arise. We suspected that it would not because we had a total sample size of 144 aqueous specimens and conducted at least three repeats of each run. The difference then seemed not to be sufficiently large that by extra numbers, it would show clearly beyond the "noise" in our assay and the natural biovariability in the specimens.

The cataract aqueous served as our nonglaucomatous controls. But was its chemoattraction substantially similar or perhaps even greater than "normal" human aqueous? It was for this reason that we also included normal bovine aqueous in the present study and found that our panel of cells migrated just as well to it as to human cataractous aqueous. Only 3 of our 63 POAG specimens had cataracts requiring surgery, but they did not stand out from the rest in their ability to stimulate migration. In the absence of direct comparison, we have nothing to indicate that cataract aqueous was not a suitable control. Indeed, evidence has been published that suggests that, at least on the basis of protein composition, aqueous removed at cataract surgery is more reliable than that taken from postmortem normal eyes.[30]

Originally we put forward the hypothesis that the circulating aqueous humor is chemoattractive for trabecular meshwork cells and that migration, activated by the chemoattractants in aqueous, might account for some of the meshwork cell loss associated with aging and POAG. The present study has shown powerful human meshwork cell chemoattraction to aqueous in vitro and also demonstrated, on the evidence of dose–response migration runs and antibody neutralization, that sFn is a major, but not the only, attractant in the fluid. Based on our in vitro experimental results, it might be thought surprising that cell loss in vivo is so low in health and disease. Of course, our experimental design is simplistic and does not take into account factors that would prevent migration such as the presence of cell-to-cell junctions, cell-to-trabecular adhesion, inhibitors in the aqueous, receptor status of the meshwork cells, and many more. We do think, however, that our positive results justify further research in this area.

On the other hand, the second part of our hypothesis is that if the chemoattraction of the aqueous in POAG is more pronounced than normal, then the added migration pressure might explain why there are even fewer remaining meshwork cells in the glaucomatous outflow system. Although our results did not overwhelmingly support the proposal, they were sufficiently positive to give it some credence. Again, other factors need to be taken into consideration, not least of which is that sampling aqueous in the anterior chamber is thought to underestimate its protein content (and therefore its bioactivity) in the meshwork.[31] In addition, there may or may not be differences in receptor upregulation between POAG and normal meshwork cells that would make the former more vulnerable to migratory stimuli.[32] Future work will examine this possibility by examining primary and early passage cultures from normals and persons with POAG.

References

1. Alvarado J, Murphy C, Polansky J, Juster R. Age related changes in trabecular meshwork cellularity. *Invest Ophthalmol Vis Sci.* 1981; 21:714–727.
2. Alvarado J, Murphy C, Juster R. Trabecular meshwork cellularity in primary open-angle glaucoma and non glaucomatous normals. *Ophthalmology.* 1984;91:564–579.
3. Grierson I, Wang D, McMenamin PG, Lee WR. The effects of age and anti-glaucoma drugs on the meshwork cell population. *Res Clin Forums.* 1982;4:69–92.
4. Grierson I, Howes RC. Age-related depletion of the cell population in the human trabecular meshwork. *Eye.* 1987;1:204–210.
5. Grierson I, Hogg P. The proliferative and migratory activities of trabecular meshwork cells. *Prog Retin Eye Res.* 1995;15:33–67.
6. Grierson I. The outflow system in health and disease. *Bull Soc Belge Ophtalmol.* 1987;225:1–43.
7. Grierson I. What is open angle glaucoma? *Eye.* 1987;1:15–28.
8. Yan DB, Trope GE, Ethier CR, Menon A, Wakeham A. Effects of hydrogen peroxide–induced oxidative damage on outflow facility and washout in pig eyes. *Invest Ophthalmol Vis Sci.* 1991;32: 2515–2520.
9. Padgaonkar V, Giblin FJ, Leverenz V, Lin L–R, Reddy VN. Studies of H_2O_2-induced effects on cultured bovine trabecular meshwork cells. *J Glaucoma.* 1994;3:123–131.
10. Grierson I, Calthorpe CM. Characteristics of meshwork cells and age changes in the outflow system of the eye: their relevance to primary open angle glaucoma. In: Mills KB, ed. *Glaucoma.* Oxford: Persimmon Press; 1989:12–31.
11. Rohen JW, Van Der Zypen E. The phagocytic activity of the trabecular meshwork endothelium. *Graefes Arch Clin Exp Ophthalmol.* 1968;175:251–266.
12. Shabo AL, Maxwell DS. Observations on the fate of blood in the anterior chamber. *Am J Ophthalmol.* 1972;73:25–36.
13. Grierson I, Lee WR. Erythrocyte phagocytosis in the human trabecular meshwork. *Br J Ophthalmol.* 1973;57:400–415.
14. Grierson I, Chisholm IA. Clearance of debris from the iris through the drainage angle of the rabbits eye. *Br J Ophthalmol.* 1978;62: 694–704.
15. Calthorpe CM, Grierson I. Fibronectin induces migration of bovine trabecular meshwork cells in vitro. *Exp Eye Res.* 1990;51:39–48.
16. Calthorpe CM, Grierson I, Hitchings RA. Chemoattractants produced by ocular cells induce trabecular meshwork cell migration. *Int Ophthalmol.* 1991;15:185–192.
17. Hogg P, Calthorpe CM, Ward S, Grierson I. The migration of cultured bovine trabecular meshwork cells to aqueous humor and constituents. *Invest Ophthalmol Vis Sci.* 1995;36:2449–2460.
18. Reid T, Kenney C, Waring GO. Isolation and characterization of fibronectin from bovine aqueous humor. *Invest Ophthalmol Vis Sci.* 1982;22:57–61.
19. Kenney MC, Lewis W, Redding J, Waring GO. Decreased fibronectin levels in aqueous humor after corneal injury. *Ophthalmol Res.* 1986;18:165–171.
20. Polansky JR, Weinreb RN, Baxter JD, Alvarado J. Human trabecular cells, I: establishment in tissue culture and growth properties. *Invest Ophthalmol Vis Sci.* 1979;18:1043–1049.
21. Grierson I, Marshall J, Robins E. Human trabecular meshwork in primary culture: a morphological and autoradiographic study. *Exp Eye Res.* 1983;37:349–365.
22. Grierson I, Kissun R, Ayad S, et al. The morphological features of bovine meshwork cells in vitro and their synthetic activities. *Graefes Arch Clin Exp Ophthalmol.* 1985;223:225–236.
23. Alvarado J, Wood I, Polansky JR. Human trabecular cells, II: growth pattern and ultrastructural characteristics. *Invest Ophthalmol Vis Sci.* 1982;23:464–478.
24. Pang I–H, Shade DL, Clark AL, Steely HT, DeSantis L. Preliminary characterisation of a transformed cell strain derived from trabecular meshwork. *Curr Eye Res.* 1994;13:51–63.
25. Martin–Vasallo P, Ghosh S, Coca–Prados M. Expression of Na,K-ATPase alpha subunit isoforms in the human ciliary body and cultured ciliary epithelial cells. *J Cell Physiol.* 1989;141:243–252.
26. Tripathi RC, Bosiruth NSC, Li J, Tripathi BJ. Growth factors in the aqueous humor and their clinical significance. *J Glaucoma.* 1994; 3:248–258.
27. Ellis I, Grey AM, Schor AM, Schor SL. Antagonistic effects of TGF-beta 1 and MSF on fibroblast migration and hyaluronic acid synthesis: possible implications for dermal wound healing. *J Cell Sci.* 1992;102:447–456.
28. Jampel HD, Roche N, Stark WJ, Roberts AB. Transforming growth factor-beta in human aqueous humor. *Curr Eye Res.* 1990;9:963–969.
29. Burke J, Foster S, Herschler J. Aqueous humor as a modulator of growth in fibroblast cultures. *Curr Eye Res.* 1983;2:835–841.
30. Tripathi RC, Millard CB, Tripathi BJ. Protein composition of human aqueous humor: SDS-PAGE analysis of surgical and post-mortem samples. *Exp Eye Res.* 1989;48:117–130.
31. Freddo TF. The Glenn A. Fry Award Lecture 1992—aqueous humor proteins: a key for unlocking glaucoma? *Optom Vis Sci.* 1992;70:263–270.
32. Tripathi RC, Yang RC, Borisuth NSC, Tripathi BJ. The role of receptors in the trabecular meshwork as targeted to the development of antiglaucoma therapy. *Drug Dev Res.* 1992;27:191–228.