Migration of Cultured Bovine Trabecular Meshwork Cells to Aqueous Humor and Constituents

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Purpose. To investigate the migration of cultured bovine trabecular meshwork cells to aqueous humor and some of its constituents, and to compare the migration of normally proliferating and growth-arrested cells.

Methods. Cellular migration was evaluated in 48-well microchemoattraction chambers, and the chemoattractants used were bovine aqueous humor, glycoproteins, and growth factors.

Results. The meshwork cells responded well to bovine aqueous humor, and antibody neutralization experiments showed that fibronectin accounted for approximately 40% of aqueous chemotraction. The glycoproteins laminin, thrombospondin, and transferrin elicited only modest migratory activity. Platelet-derived growth factor was the most powerful chemoattractant of the growth factors tested, and the others produced moderate migratory effects. Basic fibroblast growth factor was not chemoattractive on its own but stimulated migration when combined with heparin. Growth-arrested cells showed less migration to a standardized chemotactic stimulus than did proliferating meshwork cells.

Conclusions. For the first time, aqueous humor was shown to act as a migratory stimulus for meshwork cells in vitro. The major attractant is fibronectin; the remaining active constituents must still be identified.

An important feature of the aging outflow system is a progressive loss of trabecular meshwork cells from all regions.1–4 The loss also occurs in nonhuman primates5 and nonprimate species.5 Some authorities consider that many other age-related alterations to the structure, and particularly to the extracellular matrix composition of the meshwork,5,7–9 are direct or indirect consequences of the progressive loss of meshwork cellularity.1–4

In primary open-angle glaucoma, where extracellular matrix changes are thought to be more marked than in age-matched healthy subjects, cell numbers are depressed to a substantially greater degree than are those occurring with increasing age.2,10,11 Clearly, an appreciation of the dynamics of meshwork cell survival in the outflow system is fundamental to our understanding of age-related changes and glaucomatous disease at this site. The mechanism of cell loss is still obscure, but several hypotheses exist that need to be tested.

The mechanism for replacing cells that are lost from the outflow system appears to be severely curtailed, but why are cells lost in the first place? Undoubtedly cells have a finite life span and their survival could be influenced by wear and tear or by toxins in the aqueous humor, and both explanations have been proposed. However, meshwork cells are migratory for several species, including man,12–18 and this would be a nonlethal mechanism to account for cell depletion. Actively locomotory meshwork cells have been shown to leave their native trabeculae, pass through the intertrabecular spaces, and enter Schlemm’s canal by negotiation with the canalicular endothelium.12–15 Although it has not been demonstrated as a route for meshwork cells to leave their trabecular location, the interstitial spaces of the ciliary muscle are a possible second migratory pathway.

The stimuli that provoke meshwork cell migration remain obscure, but it is our hypothesis that soluble factors present in the aqueous humor act as chemoattractants for meshwork cells. Indeed, we showed in a
previous study that soluble fibronectin (sFn) is a potent motogen for cultured bovine meshwork cells, and sFn is present in both bovine and human aqueous at levels that produce a substantial response in our migration assays. Cultured bovine meshwork cells have phenotypic and many functional similarities to their human equivalent, and cell-surface characteristics also compare favorably. The bovine meshwork in culture provides an inexpensive source of useful target cells. Bovine aqueous humor also can be tapped in large quantities, and the supply of eyes is not limited.

The object of the present study was to determine whether soluble growth factors, known to be present in the aqueous humor, affect meshwork cell migration, particularly for the concentrations at which the factors are found in aqueous humor. In addition, we wanted to determine the type of migratory response that is elicited by the most effective chemoattractants. However, the main goal of this study was to determine whether aqueous humor evokes a migratory response from meshwork cells and so test whether the fluid may play a role in cellular attrition from the meshwork.

MATERIALS AND METHODS

Meshwork Culture

Bovine eyes came on ice from a local abattoir and were dissected within 8 hours of reaching our laboratory, but delays of up to 24 hours had little effect on growth in culture. Whole eyes were immersed in 70% ethyl alcohol for a few seconds. The anterior segments were removed, the lenses extracted, and the tissue soaked in a solution of penicillin and streptomycin (100 units/ml of phosphate-buffered saline) for 30 minutes. Subsequently the outflow tissues were exposed under a dissecting microscope in a sterile hood.

Bovine meshwork cells were isolated and cultured according to a technique first described in our laboratory. The explants were placed in 25-cm² flasks and fed with minimal essential medium (MEM) supplemented with 10% newborn calf serum (Gibco BRL Life Technologies, Paisley, UK). The flasks were incubated at 37°C in a 5% CO₂ atmosphere and left undisturbed for 1 to 2 weeks. Explants were removed when primary outgrowths were established and the cells were fed twice per week with culture medium. At confluence, the cells were detached from their flasks with 1 ml 50:50 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (Gibco), the reaction terminated with MEM containing 10% serum, and the cells passaged at a split ratio of up to 1:8. Bovine meshwork cells were harvested to third passage and either used in the migration studies or frozen down and stored in liquid nitrogen.

There are no clear-cut markers to identify meshwork cells in vivo or in vitro. As a result, several routine quality-control checks were performed in accordance with our previous study. The bovine meshwork was large compared with other species, so all dissections were made well within its boundaries. Sample explants of bovine meshwork and explants removed from culture were processed for histologic analysis, sectioned, and examined periodically for purity. In addition, the limbal and angular tissue from which the dissection was made was processed frequently either for histologic or scanning electron microscopic examination to check the bed from which the explant was taken.

Meshwork Chemotaxis

Bovine meshwork cells between third and fifth passage were used for the migration assays. It in our previous studies on these cells, we showed that they have a stable phenotype and growth curve at least up to seventh passage. The migration studies were conducted using 48-well microchemoattraction chambers (Neuro Probe, Cabin John, MD), as previously described by Calthorpe and coworkers. An appropriate attractant was made up to the required range of concentrations in MEM and then placed into the 25-µl-capacity lower wells using a micropipette. A polycarbonate membrane of either 8- or 10-µm-diameter pore size (Nucleopore, Pleasonton, NJ), with a rubber gasket surround, covered the 48 lower wells. The section containing the 48 upper wells was placed on top and secured (Fig. 1). The membranes were available with and without the wetting agent polyvinyl-pyrollidone.

The settlement of our cells to membranes containing the wetting agent was poor; however, if coated with porcine gelatin type 1 (300 bloom at 5 mg/l; Sigma Chemical Co, St. Louis, MO), the settlement and migration (unpublished observations) was indistinguishable from that on the membranes without the wetting agent. Both types of membrane were used in this investigation, but in experiments involving several chambers run at once, only membranes of the same type and batch were considered.

Preconfluent or just confluent cultures, where the meshwork cells were still in log phase of growth, were detached from their flask with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid. The enzymic treatment was terminated by adding 10% newborn calf serum in MEM. The cells were formed into pellets.
FIGURE 1. Diagrams to show (A) top and (B) side views of the 48-well microchemoattraction chamber used in the migration studies.

in a bench centrifuge at 100g for 10 minutes, thoroughly washed and resuspended in serum-free MEM, and their numbers counted in a Coulter counter (ZF, Coulter Electronics Ltd, Luton, UK). A volume of 50 µl of serum-free MEM plus 40,000 cells was placed into each of the upper wells using a pipette (Fig. 1). The whole apparatus was incubated in 5% CO₂ at 37°C for 4 hours; then it was opened and the membrane was removed, placed in ethanol for 15 seconds, and air dried. After staining with hematoxylin for 30 minutes, the membranes were washed in tap water and then mounted on large glass slides. The number of settled cells on the top surface and migrated cells on the under surface of the stained membranes was determined by counting nuclei in 20 high-power fields (×1000) per well using a light microscope (Vickers Instruments, UK). The settled and migrated cells were counted separately; to differentiate clearly between them, the membranes were mounted with the settled layer of the membrane containing many darkly stained, rounded cells and the migrated cells were more sparsely distributed and spread in profile.

Zigmond Hirsch checkerboard analysis was done on those substances that produced a positive chemotactic response from the bovine meshwork cells. The checkerboard assay was done to identify the type of response elicited. Soluble chemoattractants can produce either a directional, gradient-dependent response (chemotaxis) or a nondirectional, random, gradient-independent stimulation (chemokinesis). Briefly, this involves establishing a range of concentrations of the potential attractant, not only in the lower wells but also in the upper wells that contain the cells to be stimulated. The checkerboard establishes a series of wells containing an increasing gradient of stimulus (chemotaxis) and an increasing concentration with no gradient (chemokinesis).

Results were expressed either as the number of meshwork cells migrated per unit area or the migrated cells expressed as a percentage of the settled cells. The number of cells counted in each high-power field ranged from 0 to 40, depending on the chemoattractants and the cell responses. Twenty high-power fields represented 0.54 mm² or 1/15 of the area of each well. Each assay was performed at least three times and repeated on three separate occasions.

Intraobserver error for the cell counts was measured by the same observer counting the same wells a second time, and interobserver error was measured by an experienced second observer counting the same membrane again. The percentage error for intra- and interobserver counts was calculated and ranged from 3% to 5% for intraobserver error and from 8% to 10% for interobserver error.

Chemoattractants
Bovine sFn was a powerful stimulus for meshwork cell migration in previous investigations, and it served as a positive control and reference standard throughout the study. In each experiment, sFn, at its optimum chemoattractive concentration of 30 µg/ml in MEM, was run as a point reference for cross-comparison. The negative control was serum-free MEM; in some experiments, a 1% fetal calf serum (FCS) solution in MEM was used to provoke meshwork cell migration. Preliminary investigations showed that 1% FCS was a reliable chemoattractant for meshwork cells, and it was as effective as 20 to 30 µg/ml sFn (data not shown).

The following potential stimulants were investigated:

- Laminin; range, 1 to 400 µg/ml
- Thrombospondin; range, 0.0001 to 500 µg/ml
- Transferrin; range, 0.1 to 500 µg/ml
- Calmodulin; range, 10 to 1000 ng/ml
- Platelet-derived growth factor (PDGF), containing isoforms AB, BB, and AA; range, 25 to 100 ng/ml
- Epidermal growth factor; range, 2 to 20 ng/ml
Fibroblast growth factor (FGF) containing both acidic and basic forms; range, 50 to 1000 ng/ml
Acidic fibroblast growth factor; range, 50 to 1000 ng/ml
Basic fibroblast growth factor; range, 50 to 1000 ng/ml
Basic fibroblast growth factor (100 ng/ml) combined with heparin (200 ng/ml)
Transforming growth factor beta containing isoforms 1 and 2; 0.1 to 50 pg/ml
Insulin-like growth factors 1 and 2; range, 1 to 100 ng/ml.

All stimulants were purchased from Sigma Chemical Company, and purity was checked by gel electrophoresis.

**Aqueous Humor**

Bovine eyes collected from the abattoir were rushed to the laboratory on ice; the globes were washed in antibiotic solution (penicillin and streptomycin, 100 units/ml, and under a dissecting microscope in a sterile hood, between 500 and 700 μl aqueous humor was removed from the anterior chamber of each eye. Aqueous humor was removed using a 1-ml syringe with a short (gauge 25) needle, care being taken not to deform the anterior chamber and to avoid touching the iris. In addition, sections of the entire anterior segment up to the pars plana posteriorly were processed for histologic analysis and sectioned. The ciliary processes were examined for any signs of swelling or disruption to indicate possible damage to the blood-aqueous humor barrier.

The aqueous samples were transferred to silicon-treated microtubes (silicon prevents sticking of sFn and other glycoproteins), labeled, and frozen to −70°C until needed. Testing showed that there was no obvious loss of chemoattractivity with storage as long as 6 months. All specimens reported in this study were stored for less than 6 months. Migration experiments with aqueous humor were repeated at least 10 times.

A standard double-antibody enzyme-linked immuno-sorbsent assay, as previously described by Wilson-Holt and coworkers in 1992 using a fibronectin rabbit anti-human primary antibody (Dako, High Wycombe, UK) and rabbit anti-sFn conjugated to peroxidase as a secondary antibody (Dako), was done to determine the concentration of sFn in aqueous humor. To estimate the contribution made by sFn to aqueous humor’s chemoattractivity, an affinity-purified polyclonal antibody against human plasma sFn (Sigma) was used to neutralize sFn activity. The amount of antibody needed for neutralization was determined from agarose plates (Pharmacia, Milton Keynes, UK) and used to neutralize sFn activity. The amount of antibody needed for this process was determined from agarose plates (Pharmacia) in which a central well containing a known concentration of sFn was surrounded at a distance of 1 cm by wells containing differing concentrations of antibody (Auchterloony assay). The antibody was as effective against bovine as against human sFn; from the line of equivalence, it was evident that the antibody needed to be five times the concentration of antigen for complete equivalence. Based on these findings, antibody for neutralization experiments was added at 10 times the aqueous sFn concentration found from the enzyme-linked immunosorbent assay. Samples of bovine aqueous humor (100 μl) were boiled for 2 minutes in sealed plastic containers. Treatment of this type denatures sFn and eliminates sFn chemoattraction.

**Proliferation Control**

The migratory responsiveness of cultured meshwork cells in the log-phase of growth, which was the normal status of cells in chemoattraction experiments, was compared with that of growth-arrested meshwork cells, the normal condition of these cells in vivo. Growth arrest was achieved either using sodium butyrate or suspending the cells in serum-free medium. Sodium butyrate has been used to inhibit division in other ocular cell types and does so without significant toxic effects. It was introduced into the culture medium for periods as long as 3 days at concentrations not exceeding 2 mM; the cultures were photographed by phase-contrast optics before and after treatment. The effect of butyrate on proliferation was assessed either by a two-step proliferation assay adapted from Campochiaro and Glaser or by bromodeoxyuridine incorporation and subsequent immunohistochemical analysis.

The two-step proliferation assay measured changes in cell numbers effected by adding sodium butyrate to the cells’ culture medium and was done in the following manner. Bovine meshwork cells were seeded into each well of a 24-well plate at a concentration of 20,000 cells/ml of culture medium (MEM containing 10% newborn calf serum). The cells were allowed to settle for 24 hours at 37°C in a humidified 5%CO₂/95% air incubator. After 24 hours, the cells in the first column of four wells were washed with phosphate-buffered saline, removed from the wells with trypsin/ethylenediaminetetraacetic acid solution, and counted using a Coulter counter. The cell counts obtained were used as the base line with which to compare future counts. The medium was removed from the remaining five columns, and control medium (MEM with 10% newborn calf serum) containing different concentrations of sodium butyrate (0, 0.5, 1, 1.5, 2 mM) was added. The plate was incubated for 5 days, after which the cells were removed and counted as above.
To assess the effect of sodium butyrate on DNA synthesis, experiments were done using bromodeoxyuridine, a thymidine analogue that is incorporated into S-phase of the cell cycle. Bovine meshwork cells were seeded into the detachable wells of eight-well Lab-Tek slides (Gibco BRL Life Technologies). Bromodeoxyuridine was added to the control and butyrate-treated wells as a 0.1% solution in culture medium (Cell Proliferation Kit, Amersham International, Amersham, UK) and left for 12 hours. The cultures were washed in phosphate-buffered saline and fixed in 95% ethanol/5% acetic acid for 30 minutes. The S-phase nuclei, having incorporated bromodeoxyuridine, were detected using avidin/biotin immunoperoxidase labeling with dimethyl-aminobenzaldehyde to produce a stained end product. Counterstaining was with Light green (Gurr Certistain, Merck, Poole, UK). Counts of positive and negative nuclei were made with the X40 objective of a light microscope (Polyvar-Reichert, Leica, Milton Keynes, UK). On average there were 300 cells in each field, five fields were counted per well, and this sample covered 1/50 of the total area of each Lab-Tek well. Five experiments were performed at each concentration of butyrate. Intra- and interobserver errors were accounted for in the same way as for the migration experiments. Intra- and interobserver errors were 5% and 10%, respectively.

Mechanical arrest of cell division involved trypsinizing preconfluent meshwork cells off their flasks, forming cells into pellets by centrifugation (see previous description), washing in MEM, and resuspending in MEM for as long as 4 hours with gentle agitation. Chemical and mechanical growth-arrested cells were compared with control preconfluent meshwork cells for their ability to migrate through the permeable membrane of a microchemotaxis chamber in response to a 1% FCS stimulus. Trypan blue exclusion showed that the cells remained viable for this period; if plated out, however, they had a protracted lag phase before log-phase growth when compared with normal cultures that had been treated with trypsin but not maintained in suspension.

All the data were analyzed using the Student’s t-test. When required, the Bonferroni correction was used to eliminate spurious significant differences generated by multiple comparisons.

RESULTS

In line with our previous studies, the bovine meshwork cells produced a marked migratory response to sFn (optimum, 20 to 30 μg/ml). The glycoproteins laminin, thrombospondin, and transferrin were also chemoattractive to meshwork cells but were not as effective as sFn. Laminin did not become significant above background migration until a concentration of 5 μg/ml was achieved (P < 0.01) (Fig. 2). The optimum response was produced by 75 to 100 μg/ml, but this was about one half the response produced by the optimum concentration of 50 μg/ml soluble fibronectin (mean = solid line; SEM = dotted lines).

PDGF produced a massive migration of the meshwork cells in the test range of 25 to 100 ng/ml, with an optimum reaction to 50 ng/ml (Fig. 3A). The PDGF effect was significantly greater than the optimum sFn response (P < 0.001) at 25 and 50 ng/ml, the latter being approximately two thirds greater than the positive control (Table 1) and 12 times greater than background (Fig. 3A). Zigmund Hirsch checkerboard analysis of the meshwork cell migration to PDGF showed that chemotaxis (first column) and...
TABLE 1. Optimum Concentrations and Relative Chemoattractivity of Substances Under Investigation

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Optimum Concentration for Migration</th>
<th>Percent Migration*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF (70% AB, 30% BB)</td>
<td>50 ng/ml</td>
<td>170.9</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>20–50 µg/ml</td>
<td>100</td>
</tr>
<tr>
<td>FGF (a and b)</td>
<td>100 ng/ml</td>
<td>58.2</td>
</tr>
<tr>
<td>Laminin</td>
<td>75–100 µg/ml</td>
<td>49.3</td>
</tr>
<tr>
<td>IGF-1</td>
<td>50 ng/ml</td>
<td>44.4</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>100 ng/ml</td>
<td>37.5</td>
</tr>
<tr>
<td>Transferrin</td>
<td>10 µg/ml</td>
<td>37.0</td>
</tr>
<tr>
<td>IGF-2</td>
<td>1 ng/ml</td>
<td>29.9</td>
</tr>
<tr>
<td>bFGF + Heparin</td>
<td>100 ng/ml Heparin + 200 ng/ml</td>
<td>25.6</td>
</tr>
<tr>
<td>aFGF</td>
<td>100 ng/ml</td>
<td>21.2</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>100 µg/ml</td>
<td>15.5</td>
</tr>
<tr>
<td>TGF-β (~1 and ~2)</td>
<td>0.1–10 pg/ml</td>
<td>9.8</td>
</tr>
<tr>
<td>EGF</td>
<td>10 ng/ml</td>
<td>4.0</td>
</tr>
<tr>
<td>bFGF</td>
<td>100 ng/ml</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*The response has been calculated as the percent migration of bovine meshwork cells compared to an optimum concentration of fibronectin.

PDGF = platelet-derived growth factor; FGF = fibroblast growth factor; IGF = insulin-like growth factor; bFGF = basic FGF; aFGF = acidic FGF; TGF-β = transforming growth factor beta; EGF = epidermal growth factor.

chemokinesis (diagonal) occurred (Fig. 3B). The chemotaxis and chemokinesis were of the same order at 10 and 25 ng/ml, but chemotaxis was significantly greater than chemokinesis (P < 0.01) at 50 ng/ml of PDGF. When PDGF was absent from the lower wells (top row), migration was a log unit lower than when PDGF was present, regardless of the concentration in the upper wells (Fig. 3B).

Epidermal growth factor was a marginal chemoattractant for meshwork cells in the range 2 to 25 ng/ml, but the migrational reaction to all these concentrations was feeble when compared with sFn (Table 1). Concentrations greater than 10 pg/ml were tested and shown not to provoke migration (data not shown). Fibroblast growth factor produced an optimum response at 100 ng/ml, which was significantly greater than background and approximately two thirds of the sFn response (Table 1). The FGF we first used was a mixture of acidic and basic FGF, and the mixture was considerably more effective as an attractant than were the constituent parts. When purified acidic FGF and basic FGF were run separately, it was clear that only acidic FGF stimulated the meshwork cells and basic FGF migration was at background levels (Table 1). Heparin can increase the chemoattractivity of basic FGF to some cell types. Heparin alone had no effect on meshwork cell migration, but 100 ng/ml of basic

FIGURE 3. (A) A dose-response histogram for the migration of bovine meshwork cells to platelet-derived growth factor PDGF. Each column represents the mean (and SEM) of at least four wells, and the experiments were repeated three times. Both 25 and 50 ng/ml of PDGF produced a significantly greater migratory response (P < 0.001; Student's t-test) than optimum sFn (mean = solid line; SEM = dotted lines). (B) A Zigmund Hirsch checkerboard analysis of the type of bovine meshwork cell migration produced by PDGF. Both chemotaxis (first vertical column) and chemokinesis (diagonal column) were identified. Each box shows the mean ± SEM of three determinations, and the checkerboard was repeated three times. At 50 ng/ml PDGF, chemotaxis was significantly greater than chemokinesis (P < 0.01; Student's t-test), but at lower concentrations there was no significant difference.
FGF with 200 ng/ml heparin made the growth factor a marginal but significant attractant ($P < 0.05$), comparable to acidic FGF (Table 1). Insulin-like growth factor type 1 had a moderate chemoattractive effect, approximately one half the sFn response, at 50 ng/ml; insulin-like growth factor type 2 was slightly more modest at 1 ng/ml ($P < 0.05$ against background) (Table 1).

Bovine aqueous humor was a potent stimulus for inducing meshwork cell migration (Fig. 4A). Given the importance of establishing the chemoattractive nature of aqueous humor, we ran these experiments at least 10 times and produced similar but not identical results. In all runs, the optimum aqueous response was at least as good as and often better than optimum sFn. At 50 μl/ml, the lowest concentration studied in these experiments, the migration was significantly higher than background ($P < 0.001$ or better for each run). The migration peaked between 200 and 400 μl/ml; the example shown has an optimum of 200 μl/ml (Fig. 4A). Other runs that concentrated on the peak range showed that the differences between 200, 300, 400, and 600 μl/ml were marginal and clearly represented a plateau (seen to some extent in the first vertical column of the Zigmond Hirsch checkerboard (Fig. 4B). The checkerboard showed also that chemotaxis and chemokinesis were equally prominent components of the migratory response to aqueous humor (Fig. 4B).

Analysis of 10 samples of bovine aqueous humor by enzyme-linked immunosorbent assay yielded an sFn content that ranged from 1.30 to 3.30 μg/ml, with a mean and standard error of the mean of 2.35 ± 0.2 μg/ml. The migration response to 400 μl/ml bovine aqueous humor, which was a concentration at the far limit of the dose optimum plateau, in line with previous experiments, was seen to be a marginally more effective migrational stimulant than optimum sFn and FCS ($P < 0.05$, not significant) (Fig. 5). Boiling aqueous reduced the activity by more than one half, but a significant migratory component above background was still present ($P < 0.01$). The sFn level in 400 μl/ml aqueous humor (40% of the mean sFn level in bovine aqueous humor, or approximately 0.9 μg/ml) produced a significant chemotraction ($P < 0.01$), but it also accounted for less than one half of the aqueous migratory activity. The introduction of excess
anti-sFn antibody to an estimated sFn level in 400 µl/ml aqueous humor (9.0 µg/ml and 0.90 µg/ml) reduced the migration to background levels, whereas a similar introduction of anti-sFn antibody to 400 µl/ml aqueous humor reduced migration to about one half (Fig. 5). Based on this experiment, aqueous humor seems to be an effective chemoattractant for meshwork cells. Approximately 50% of the chemoattraction may be due to fibronectin; of the other 50%, most is produced by a substance or substances whose chemoattractivity is not compromised by heat.

All results to this point were produced using proliferating meshwork cells as the test system. When meshwork cells were exposed to increasing concentrations of as much as 2 mM sodium butyrate for 3 days, they remained viable (95% excluded trypan blue, which was the same as control, untreated cells) but proliferation was depressed (Fig. 6). Two-step growth assays showed that after 3 days, control cells multiplied from 40,000 to more than 200,000 (a 5.5-fold increase), but the presence of 1 mM sodium butyrate reduced the growth to 100,000 cells and 2 mM sodium butyrate reduced it to approximately 75,000 cells (less than a twofold increase). Immunohistochemical staining to identify bromodeoxyuridine-labeled nuclei showed that nearly one half incorporated the S-phase marker in control cultures, but incorporation was less than 10% after 3 days' treatment with sodium butyrate. The drug-induced steady decrease in the rate of growth and DNA synthesis of meshwork cells was paralleled by a decrease in migrational responsiveness of these cells (Fig. 7). Nearly 25% of normal meshwork cells migrated to 1% FCS, whereas only 15% of growth-arrested cells migrated to the same stimulus (P < 0.001) (Fig. 7).

The alternative mechanism to arrest growth of the meshwork cells was to maintain them in suspension for 3 hours in serum-free conditions. Suspension for this period did not significantly affect trypan blue exclusion, did not adversely affect settlement on the migration membranes, but did have a substantial inhibitory effect on migration. Migration of control meshwork cells and meshwork cells whose growth was arrested by suspension was significantly greater than background (P < 0.01), but the migration of growth-arrested cells was a quarter of the control values (P < 0.001) (Fig. 8).

**DISCUSSION**

Our choice of sFn as the positive control, apart from the fact that it was previously shown to be a meshwork
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0.5 0.1 1.5
Sodium Butyrate [mM]

0 100 200 300 400
Cell Number

0 10 20 30 40
% Migration

FIGURE 7. A combined dose-response curve and histogram to show the decrease in migration of bovine meshwork cells after 3 days of incubation with sodium butyrate. The histogram (left vertical axis) shows a proportionate decrease in cell numbers migrated with increasing concentrations of sodium butyrate. Each column represents the mean (and SEM) of four wells, and the experiment was repeated three times. The line graph (right vertical axis) also shows a concomitant decrease in percentage of cell migration.

cell migratory stimulant, was made for various reasons. Fibronectin is synthesized by both human and bovine meshwork cells in culture, seems to be an integral component of the extracellular matrix of the trabeculae, and is present in aqueous humor. The sFn content of bovine aqueous humor that we calculated to be 2.35 ± 0.20 /μg/ml was remarkably similar to the mean value produced by Reid et al for the same species (2.46 /μg/ml). Such concentrations are sufficient to evoke substantial migration from cultured meshwork cells but are 10-fold lower than the dose optimum used in the present study.

Laminin also would seem to be a well-established constituent of the outflow system, although there is some controversy about its precise location in the tissue. Some authors identified laminin in the trabeculae by immunohistochemical analysis, but Marshall et al were convinced it was absent from the trabeculae, although they did locate laminin beneath the endothelium of Schlemm’s canal. The best migratory response produced by laminin was less than one half that of sFn and, although the concentration (if any) of laminin in aqueous humor is not known, required such a large amount (75 to 100 /μg/ml) that it is hard to imagine it influencing meshwork cell mobility in the healthy eye.

Transferrin also stimulated migration to a slightly lesser degree than did laminin, but only at concentrations considerably greater than those found in aqueous humor. The high-molecular-weight glycoprotein thrombospondin has been identified in the aqueous outflow pathways, where it is thought to be locally synthesized. It stimulates the migration of several cell types, but in the present investigation it was ineffective on bovine meshwork cells. It produced a significant level of attraction at 100 /μg/ml (Table 1), a dose optimum similar to that which induces mesangial cell migration, but for meshwork cells the response is only 15% of that to sFn.

Several of the substances tested, including sFn and other glycoproteins noted previously, have been identified as components of normal aqueous humor (Table 2). However, the most potent migratory stimulant, PDGF, is only present in aqueous humor in minuscule concentrations; because it is abundant in plasma and in platelets, however, it is probably present in diseases in which inflammation occurs and the blood-aqueous barrier is disrupted.

Based on our findings to date, we predicted that normal aqueous humor would be, at best, a modest chemoattractant for meshwork cells. After all, sFn was present in aqueous humor at suboptimal levels and the peptide growth factors in the aqueous humor were, at best, marginal attractants. Therefore, it was interesting to find that aqueous humor was as good and sometimes better than optimal sFn at stimulating meshwork cell migration. Chemotaxis and chemokinesis were both components of this migrational response.

FIGURE 8. A histogram to show the effect of mechanical growth arrest on migration. After 3 hours’ suspension in serum-free medium, the migration of the growth-arrested cells was approximately one quarter of the control (P < 0.001; Student’s t-test). Each column represents the mean (and SEM) of six wells, and the experiment was repeated three times.
TABLE 2. Constituents of Aqueous Humor Tested in the Migration Studies

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Concentration in Aqueous</th>
<th>Species</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>2.4 μg/ml</td>
<td>Bovine</td>
<td>Reid et al, 1982</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>Present in trabeculae</td>
<td>Human</td>
<td>Tripathi et al, 1991</td>
</tr>
<tr>
<td>Tranferrin</td>
<td>1.4 mg/dl</td>
<td>Human</td>
<td>Tripathi et al, 1990</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Not reported</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PDGF (70% AB, 30% BB)</td>
<td>0.3 ng/ml</td>
<td>Human</td>
<td>Tripathi et al, 1994</td>
</tr>
<tr>
<td>EGF</td>
<td>0.88 ng/ml</td>
<td>Human</td>
<td>Parellman et al, 1990</td>
</tr>
<tr>
<td>TGF-β1 and −β2</td>
<td>0.2–1.0 ng/ml</td>
<td>Human</td>
<td>Granstein et al, 1990</td>
</tr>
<tr>
<td>aFGF</td>
<td>35 pg/ml</td>
<td>Bovine</td>
<td>Caruelle et al, 1989</td>
</tr>
<tr>
<td>bFGF</td>
<td>0.7–1.4 ng/ml</td>
<td>Human</td>
<td>Tripathi et al, 1988</td>
</tr>
<tr>
<td>IGF-1</td>
<td>1.4 ng/ml</td>
<td>Human</td>
<td>Castro et al, 1990</td>
</tr>
<tr>
<td>IGF-2</td>
<td>Not reported</td>
<td>Human</td>
<td>Tripath et al, 1991</td>
</tr>
</tbody>
</table>

PDGF = platelet-derived growth factor; FGF = fibroblast growth factor; IGF = insulin-like growth factor; bFGF = basic FGF; aFGF = acidic FGF; TGF-β = transforming growth factor beta; EGF = epidermal growth factor.

It was clear from the aqueous dose-response curve that sFn could not be the only constituent influencing the migratory behavior of our cells. The sFn content ranged from 1.30 to 3.30 μg/ml in the aqueous humor we sampled, so because 20 to 30 μg/ml produces the optimal migratory response, it might be expected that if sFn predominated then the migratory effect would progress with increasing concentrations to 100%. This was far from the case because, in each run, the net migratory action either stabilized or decreased after 40% aqueous humor was reached. Yet at 40%, the sFn content is less than 1 μg/ml. Using anti-sFn, neutralizing antibodies showed that sFn comprised less than one half of aqueous activity, but what of the rest if no other aqueous constituent is particularly responsive?

That migratory activity remained after short-term boiling confirms that a constituent other than a glycoprotein is chemoattractive for meshwork cells because the bioactive sites on most glycoproteins are heat labile. Some of the low-molecular-weight peptide growth factors retain their bioactivity after heat exposure, and PDGF is an obvious example. However, as already stated, PDGF is only likely to be present in substantial amounts in aqueous humor after the blood–aqueous barrier is disrupted. It is reasonable to consider that slaughtering might have traumatized the bovine eye, leading to barrier breakdown and PDGF influx. Histologic examination of the ciliary processes, however, showed no evidence of process swelling or barrier disruption. Indeed, if the barrier had been compromised to any great extent we would have expected far higher sFn and protein levels than we measured. Bours considered that abattoir death did not compromise the bovine blood aqueous barrier, and our observations seem to accord with this. The heat-stable meshwork cell motogens in bovine aqueous humor require further study, and it appears that they may have also been highlighted by Joseph et al, who used rabbit and human Tenon’s fibroblasts as the test cells in their experiments.

A question still needs to be addressed: If aqueous humor is such a good chemoattractant for meshwork cells, why are there any of them left as they continuously bathe in the fluid? Could the migratory responsiveness of meshwork cells to a range of agents, including aqueous humor, be biologically irrelevant? After all, to use another system as an example, vascular endothelial cells are exposed to a plethora of motogens in the plasma, but they fail to respond to these except in injury or disease. Presumably the adhesive and junctional modifications of vascular endothelial cells are such that they can resist chemotraction. Equally or perhaps more important is the status of the endothelium, and we know that they divide very slowly in vivo if at all. Low rates of cell division are associated with receptor downregulation, and this also would make the endothelium less likely to be influenced by an attractant. In its turn, the meshwork cell in situ has a basement membrane to cling to, and gap junctions between cells are well developed. However, division of meshwork cells in the normal eye would seem to be marginal or even nonexistent Our crude experiments taking the cells out of the division cycle, either with sodium butyrate or by maintaining them in suspension, dramatically depressed their ability to migrate to a standard stimulant.

If meshwork cells need to be in the cell cycle to be responsive to a chemoattractant, then their G0/G1 status in situ, downregulation of appropriate receptors, and strong cell-to-cell and cell-to-extracellular matrix adhesion will maintain the cell population against migratory pressures. With this triple protection, the meshwork cells will not dissipate rapidly, but they remain prone to gradual attrition. The working hypothesis we have developed is that meshwork cells cannot, once lost, be replaced because the cells that
would effect the replacement, that is those that go into the cell cycle, are the very cells that are responsive to chemoattractants in the aqueous humor. These proliferating cells tend to be lost from the trabecular meshwork and hence make no net contribution to cell numbers; rather quite the reverse is true. Examination of our data on age-related loss of meshwork cellularity^4 indicates that only 20 cells need be lost per day without replacement to account for the linear depreciation in numbers.

Our model would also predict that in glaucomatous diseases (such as neovascular glaucoma and some inflammatory glaucomas^5 in which the blood–aqueous barrier is compromised), this produces an influx of meshwork cell motogens (such as PDGF) and glycoproteins, which causes meshwork cell numbers to plummet. As yet, no histopathologic evidence exists to confirm or dismiss the prediction. Current work in our laboratory, using human meshwork cells and aqueous humor, focuses on some of the issues discussed.

**Key Words**
aqueous humor, bovine meshwork cells, fibronectin, glycoproteins, growth arrest, growth factors, migration

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