Effect of Matrix Metalloproteinases Activity on Outflow in Perfused Human Organ Culture

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PURPOSE. To test the hypothesis that extracellular matrix turnover, mediated by the matrix metalloproteinases, modulates aqueous humor outflow facility in a human outflow model.

METHODS. Matrix metalloproteinase activity was manipulated and outflow facility evaluated using perfused human anterior segment organ culture. Purified matrix metalloproteinases, tissue inhibitors of metalloproteinases (TIMPs), and several families of synthetic inhibitors of matrix metalloproteinases were added to the perfusion medium. Matrix metalloproteinase expression was increased by adding recombinant interleukin (IL)-1α. Kinetic inhibition analysis was conducted for stromelysin, gelatinase A, and gelatinase B with the various inhibitors. Live-dead staining was used to evaluate culture viability.

RESULTS. Increasing metalloproteinase activity, by adding purified metalloproteinases or by inducing their expression by IL-1α treatment, increased outflow facility. Inhibition of endogenous trabecular metalloproteinase activity using TIMP or several families of synthetic metalloproteinase inhibitors reduced outflow rates. The elevation and the reduction of outflow rates were reversible, with changes requiring 1 to 3 days. Kinetic enzyme inhibition analysis produced 50% inhibitory concentration values for these inhibitors that were compatible with the concentration ranges for outflow inhibition.

CONCLUSIONS. The ability of several specific matrix metalloproteinase inhibitors to reduce outflow facility implies that endogenous extracellular matrix turnover by these enzymes was required for the maintenance of trabecular outflow resistance, at least in this human culture model. These observations provide support for the hypothesis that controlled extracellular matrix turnover is important in the regulation of aqueous humor outflow facility. (Invest Ophthalmol Vis Sci. 1998;39:2649-2658)

Normal and pathologic extracellular matrix (ECM) turnover is initiated primarily by members of a family of secreted zinc proteinases, the matrix metalloproteinases.1-7 Interstitial collagenase-1, gelatinases A and B, and stromelysin-1 have been studied in the most detail, and their partially overlapping substrate specificities include most ECM macromolecules. Additional family members have been identified, including several membrane-bound forms. The metalloproteinases are secreted as latent proenzymes and share a highly-conserved "cysteine switch" activation mechanism.8,9 A cysteine in the propeptide, which extends across the active site in a direction opposite that of substrates, serves as the fourth chelation partner of the active-site zinc. Disruption of this bond by any of several mechanisms allows a water molecule to bind the zinc, resulting in active enzyme. Physiologic activation is thought to be initiated by proteinase cascades involving plasminogen activator-plasminogen, or several members of the metalloproteinase family including the membrane-type matrix metalloproteinases, or both, although details remain to be fully elucidated. Metalloproteinase activity is constrained extracellularly by a family of tissue inhibitors (tissue inhibitors of metalloproteinases [TIMPs]).1-3-5,10 The TIMPs are secreted proteins that are specific inhibitors of the matrix metalloproteinases with Ki values in the low to subnanomolar range.19,20 TIMP 1, 2, 3, and 4 all inhibit the metalloproteinases but also serve specific regulatory roles with the various enzyme forms.12,19-24 Crystal and nuclear magnetic resonance structures of the catalytic domain of several members of the metalloproteinase family and the inhibitory domain of TIMP-2 have been solved.25-28 The binding conformations of several inhibitors within the metalloproteinase-active sites have also been established.

Glaucoma is a major blinding disease, affecting as many as 2.47 million people in the United States39-30 and as many as 66.8 million worldwide.31 An unidentified obstruction reduces aqueous humor outflow facility through the trabecular meshwork causing elevated intraocular pressure. Much of glaucomatous optic neuropathy is caused by elevated intraocular pressure.32-35 The trabecular ECM may provide much of the normal resistance to aqueous humor outflow,32,36-39 and ab-
normal ECM accumulates in the trabecular filtration region in glaucoma.\textsuperscript{57-45} Trabecular ECM turnover is relatively rapid with average glycosaminoglycan half-lives of approximately 1.5 days, compared with 1 to 2 weeks in the adjacent cornea or sclera.\textsuperscript{46} Trabecular ECM turnover seems to involve the matrix metalloproteinases and to be constrained by the TIMPs.\textsuperscript{37-39,47,48} The efficacy of a common clinical laser treatment for glaucoma seems to be related primarily to the sustained induction of stromelysin and gelatinase B, specifically within the portion of the trabecular meshwork that is thought to modulate aqueous humor outflow.\textsuperscript{50,59}

Although the trabecular ECM and aqueous humor outflow through it have been studied in detail for years, no clear and testable hypothesis has been established that provides a molecular explanation for normal or pathologic outflow pathway regulation. Until recently,\textsuperscript{51-53} glaucoma has also been refractory to genetic analysis, and its molecular origin is still unclear. Thus, to test the hypothesis that ECM turnover, initiated by the peptide collagenase inhibitor, HS-CH\textsubscript{2}-CH\textsubscript{2}-CH(CH\textsubscript{2}-CH(CH\textsubscript{3})\textsubscript{2} > were purchased from Sigma (St. Louis, MO); the mercapto-

**Materials and Methods**

Recombinant human interleukin (IL)-1\textalpha was from R & D Systems (Minneapolis, MN); minocycline and other tetracycline derivatives, 3-tropane hydroxamate, p-hydroxybenzoyl-Ala-Phe, 4-aminoephynol mercuric acetate, gelatin, and \beta-casein were purchased from Sigma (St. Louis, MO); the mercaptopeptide collagenase inhibitor, HS-CH\textsubscript{2}-CH\textsubscript{2}-CH(CH\textsubscript{2}-CH(CH\textsubscript{3})\textsubscript{2} > CO-Phe-Ala-NH\textsubscript{2}, and the peptide substrates, NFF-2 (Mca-RPKPVQ-Nva-Phe, 4-aminophenyl mercuric acetate, gelatin, and \beta-casein were purchased from Sigma (St. Louis, MO); the mercaptopeptide collagenase inhibitor, HS-CH\textsubscript{2}-CH\textsubscript{2}-CH(CH\textsubscript{2}-CH(CH\textsubscript{3})\textsubscript{2} > CO-Phe-Ala-NH\textsubscript{2}, and the peptide substrates, NFF-2 (Mca-RPKPVQ-Nva-Phe-Ala-NH\textsubscript{2}), NFF-3 (Mca-RPKPVQ-Nva-Phe-Ala-NH\textsubscript{2}), NFF-4 (Mca-RPKPVQ-Nva-Phe-Ala-NH\textsubscript{2}), and NFF-5 (Mca-RPKPVQ-Nva-Phe-Ala-NH\textsubscript{2}) were purchased from Peptides International (Louisville, KY). The small metalloproteinase proteopeptide fragments Ac-RGGVP-NH\textsubscript{2} and Ac-RGGVPD-NH\textsubscript{2} were synthesized using standard Fmoc chemistry on an ABI synthesizer (Foster City, CA), ethyl ether precipitated, and purified on a C-18 column by reversed-phase high-performance liquid chromatography; their purity and identity was analyzed by electrospray mass spectrometry (Macromolecular Resources, Colorado State University, Ft. Collins, CO). For some studies, TIMP-1, TIMP-2, stromelysin, gelatinase A, and gelatinase B were purchased from Biogenesis (Sandown, NH). Human foreskin fibroblast (HS27, CRL#1634) and human fibrosarcoma (HT-1080, CCL#121) were from the American Type Culture Collection (Rockville, MD).

**Human Anterior Segment Perfused Organ Culture Model**

Human donor eyes were obtained within 48 hours after death from the Lion’s Eye Bank, Portland, Oregon, and anterior segments, containing the undisturbed trabecular meshwork sandwiched between the intact cornea and a 15-mm rim of sclera were cultured for 7 days in stationary organ culture before perfusion.\textsuperscript{55} Eyes from patients with potentially confounding diseases were not used. Anterior segments were then mounted in a standard perfusion culture apparatus\textsuperscript{55-62} and perfused with culture medium\textsuperscript{54} but without amphotericin B, using a constant perfusion head of 10 cm (approximately 7.35 mm Hg) for 3 to 5 days, while outflow stabilized. Explants that did not stabilize between 1.5 \mu m/\text{min} and 8 \mu m/\text{min} at 7.35 mm Hg perfusion pressure after 7 days were not used. Culture was at 37°C in 100% humidity and 5% CO\textsubscript{2}-95% air. Outflow rates (Q, in microliters per minute) were determined gravimetrically to ±10 \mu l approximately every 12 hours. Normalized outflow facility (FC, in microliters per minute per millimeter of mercury perfusion pressure normalized to 100% of the pretreatment flow rate).\textsuperscript{50,65} Outflow has been shown to be through the trabecular meshwork,\textsuperscript{55,66,61} and flow rates are physiologic, that is, approximately 2.75 \mu m/\text{min}.\textsuperscript{53,54} Flow rates and trabecular cellularity are maintained for at least 3 weeks.\textsuperscript{55-50,64}

Microscopic analysis of 3- to 6-\mu m sections or confocal analysis of flatmount intact explants treated with live-dead viability stain (Molecular Probes, Eugene, OR) and viewed from the perspective of the anterior chamber was used to evaluate the condition of trabecular cells after completion of several perfusion studies. A confocal laser scanning microscope (model 900; Leica, Heidelberg, Germany), a krypton-argon laser, a simultaneous dual-channel detector, and 24-bit imaging in the Microbiology and Molecular Immunology (MMI) Core Facility (Oregon Health Sciences University) was used, as previously described.\textsuperscript{65} Confocal optical sections were taken at a depth of 20 \mu m to 50 \mu m into the central or slightly anterior meshwork. Live-dead staining after 15 minutes’ treatment with 70% methanol or 0.5% saponin in phosphate-buffered saline to permeabilize the cells was used to determine total cell numbers by allowing staining of all trabecular nuclei. In one permeabilized explant, 16 serial optical sections were taken at 2-\mu m steps to verify orientation and determine the density of nuclei at different depths into the meshwork. We could not focus sharply at the depth of Schlemm’s canal, particularly in the more posterior portions of the meshwork, because of instrument limitations: The maximum focal depth was approximately 50 \mu m.

Metalloproteinase inhibitors were dissolved in water or at 10,000 times final treatment concentrations in absolute ethanol for flow and kinetic studies, and vehicle controls were run in parallel. Except as specifically noted, all treatments were continuous exposures to the agent in the perfusion medium, beginning at treatment time 0, and the perfusion head was maintained at 7.35 mm Hg. The exception was in the individual metalloproteinase treatments using the commercial enzyme preparations (Biogenesis). Because of the expense of the enzymes, either stromelysin or one of the gelatinases was activated and injected directly into the anterior chamber by scleral paracentesis, using a 30-gauge, 0.5-in needle. In the treatments or in parallel sham control treatments with buffer there was no direct effect on outflow rates, and we could detect no leakage of perfused dyes from the paracentesis sites. Paracentesis was conducted three times at 12-hour intervals using 2 \mu g enzyme in each injection.

**Metalloproteinases and TIMPs**

Gelatinase A, gelatinase B, stromelysin, and TIMPs for some of the perfusion studies were purified from conditioned human anterior segment culture medium; no significant differences
were observed among these proteins and those purchased from Biogenesis. Culture medium was dialyzed against 50 mM EDTA in phosphate-buffered saline; proteinases were precipitated with 80% (NH₄)₂SO₄ and chromatographed on Sephadex G-100 SF (Pharmacia, Uppsala, Sweden); 5 mM CaCl₂ and 10 μM ZnCl₂ were added, and chromatography was repeated. This metalloproteinase preparation was analyzed by substrate gel electrophoresis (zymography) and by silver staining to determine purity. In some of the metalloproteinase flow studies, a 1:1:1 mixture (20 μg total) of gelatinase A-gelatinase B-stromelysin was heat-activated for 2 hours at 55°C before addition to perfusion medium. TIMP-2 was purified from hu-

**FIGURE 1.** Effect on outflow facility of changing perfusion pressure or of adding metalloproteinases. (A) After outflow rates had stabilized, the perfusion head was changed approximately every 3 days to evaluate outflow response. Perfusion head values are shown in millimeters of mercury above each equilibrated flow rate (%Qₒ). Points are mean ± SEM for n = 4 to 8 experiments. (B) After outflow had stabilized at 7.35-mm Hg perfusion head, 20 μg purified metalloproteinases (solid circles) or bovine serum albumin (open circles) was added (at treatment time 0) per milliliter perfusion medium. The metalloproteinases were an equimolar mixture of purified gelatinase A, gelatinase B, and stromelysin. Values are mean outflow facility (%Cₒ) ± ranges for two explants; P < 0.0001. (C) Purified stromelysin was injected into the perfusion chamber (2 μg per injection) three times at 12-hour intervals, as indicated by the arrows and the %Cₒ evaluated (n = 1).

**FIGURE 2.** Effects of metalloproteinase induction and TIMP-2 addition on outflow facility. (A) Recombinant human IL-1α (40 U/ml) was added to the perfusion medium at treatment time 0. Values are mean %Cₒ ± SEM of four explants; P < 0.0001. (B) Purified human TIMP-2 (50 nM) was added to perfusion medium, and outflow was monitored for 120 hours (solid circles). Medium was then replaced with TIMP-2-free medium and outflow monitored until recovery to pretreatment levels (approximately 100 hours; data not shown). Outflow facility (%Cₒ) is the mean ± SEM for three explants; P < 0.0001. In separate experiments, 50 U/ml IL-1α and 14 nM TIMP-2 were added together at treatment time 0 and outflow evaluated for 120 hours (open circles; n = 1).
man anterior segment culture medium by acidification (pH 4.0), heating to 50°C for 2.5 hours, centrifugation, and chromatography on diethylaminoethyl cellulose followed by Sephadex G-75 (Pharmacia). For kinetic studies, gelatinase A and B were purified, respectively, from human foreskin fibroblast (HS27) or human fibrosarcoma (HT-1080) cell-conditioned culture media, or both enzymes were purchased (Biogenesis).

For gelatinase A, fibroblast culture medium was subjected to gelatin-affinity chromatography, bound TIMP was eluted with acid (pH 2.0), and the gelatinase was eluted with 10% dimethyl sulfoxide and dialyzed. Gelatinase B was prepared similarly from fibrosarcoma medium, except the final dialysate was further purified on Con A-Sepharose (Sigma). Gelatinase A or B, purified as detailed earlier or purchased (Biogenesis), was activated with 1 mM 4-aminophenyl mercuric acetate at 37°C for 2 or 18 hours, respectively, followed by dialysis. Stromelysin for kinetic studies was purified from IL-1α-stimulated HS27 cell-conditioned medium by procion-red chromatography followed by gelatin-affinity chromatography or was purchased (Biogenesis). In either case, stromelysin was activated by incubation for 2 hours at 55°C.

Kinetic and Inhibition Analysis

Standard fluorescein isothiocyanate-conjugated protein substrate assays, using gelatin or β-casein for gelatinases or stromelysin, respectively, or fluorescence quench-release peptide substrate assays using NFF-3 and NFF-2, respectively, were used for kinetic and inhibition analysis. For determination of 50% inhibitory concentration (IC50), unless stated otherwise, the fluorescein isothiocyanate-conjugated gelatin or β-casein concentrations in the assays were 2.2 μM and 4 μM, respectively, and the enzyme concentrations were 20 nM, 90 nM, and 45 nM for gelatinase A, gelatinase B, and stromelysin, respectively. For Kd determinations, substrate concentrations were varied. Gelatinase assays were conducted in 50 mM Tris (pH 8.5), 150 mM NaCl, 5 mM CaCl2, and 10 μM ZnCl2 at 37°C, and stromelysin assays were conducted in 25 mM Tris (pH 7.5), 5 mM CaCl2, and 10 μM ZnCl2 at 37°C. Kinetic data analysis using weighted linear and nonlinear curve fitting was conducted using commercial software (Grafit; Erithacus, Middlesex, UK) or Leonora (Oxford University Press, Oxford, UK). The significance of outflow effects was evaluated by Student's t-test comparing pretreatment and posttreatment flow rates.

RESULTS

Effects of Perfusion Head and Perfusion with Metalloproteinases on Outflow

In most anterior segments, outflow rates stabilized within 3 to 5 days of starting perfusion and could generally be maintained for 1 or 2 more weeks. Approximately 25% of the explants studied did not stabilize within 7 days or exhibited flow rates outside of our acceptance criteria. The average flow rate was approximately 2.8 ± 0.2 μl/min (n = 50) at 7.35 mm Hg. To evaluate the effect of perfusion pressure on outflow rate, the perfusion pressure was changed approximately every 3 days and outflow rates measured at 12-hour intervals (Fig. 1A). Outflow rates stabilized within approximately 24 hours after each step and remained stable for several days. Flow rate...
changes were approximately proportional to head changes across the range evaluated.

When a mixture of exogenous purified matrix metalloproteinases (equal amounts of activated stromelysin-1, gelatinase A, and gelatinase B) were added to the perfusion medium, outflow facility increased significantly to 160% Co (Fig. 1B). A control protein, bovine serum albumin, produced no significant change in outflow facility. Sustained exposure to metalloproteinases, beyond approximately 150 to 300 hours, resulted in a slow decline to approximately 75% Co or below, suggestive of structural disorganization of the meshwork’s ECM caused by excessive proteinase action or perhaps by an attempt by trabecular cells to regain homeostasis (not shown). Similarly, exposure to high concentrations of metalloproteinases produced an initial increase in outflow facility followed by a decline to well below baseline levels (not shown).

In a separate experiment, purified stromelysin (Biogenesis), was injected through paracentesis directly into the perfusion chamber to evaluate its effect on outflow (see Methods section). Activated stromelysin (2 µg each time) was injected three times at 12-hour intervals and the effect on flow rate evaluated (Fig. 1C). Control injections of buffer without stromelysin caused no change (not shown). The three stromelysin injections produced an increase in outflow facility (Fig. 1C) to approximately 130% Co at 72 hours after the first injection, followed by a decline to approximately 75% Co at 96 hours and a rebound to approximately basal levels by 120 hours. A similar response was observed when purified gelatinase A or gelatinase B (Biogenesis) was used (not shown).

Effects of Metalloproteinase Induction by IL-1α on Outflow

We have previously shown that treatment with the cytokine IL-1α elevates trabecular gelatinase B and stromelysin expression.28 Adding recombinant human IL-1α to the perfusion medium continuously resulted in a significant elevation in outflow facility within 24 hours (Fig. 2A). This elevation was sustained for several days, whereas removal of IL-1α from the perfusion medium (not shown) allowed a gradual return to approximately 100% Co. Continued exposure to IL-1α did not maintain the high initial level of outflow (compare the first 40 hours with the second 40 hours), but allowed a modest decline followed by a modest rebound and then a slow decline (Fig. 2A). Although this somewhat sinusoidal bounce-decrease-rebound-decrease pattern was observed in all four experiments, it was not temporally synchronized between the explants; thus, only the initial relatively rapid rise and the overall pattern of elevation in percentage of Co were statistically significant (P < 0.0001; n = 4).

Effects of Perfusion with TIMP-2 and TIMP-2 Blockage of IL-1α Effects on Outflow

When we perfused with purified TIMP-2, we observed a significant and sustained decrease in outflow facility (Fig. 2B, solid circles). Removal of TIMP-2 from the perfusion medium at 120 hours allowed a return to pretreatment outflow facility within a few days (data not shown). The addition of TIMP-2 (14 nM) with IL-1α completely blocked and eventually overpowered the IL-1α-induced decrease in outflow facility (Fig. 2B, open circles).

Effects of Synthetic Inhibitors of Metalloproteinases on Outflow

We also used several different classes of metalloproteinase inhibitors to examine the possibility that these effects were caused by other molecular mechanism(s). Tetracycline antibiotics inhibit matrix metalloproteinase activity and have been used in this capacity to treat rheumatoid arthritis and periodontal disease.70 Adding minocycline to the perfusion medium produced a significant and sustained decrease in outflow facility (Fig. 3A). Removal of minocycline from the perfusion medium did not allow reversal of the outflow depression, even after several days. However, spectroscopic evaluation of minocycline levels in the medium exiting the eye showed that it was retained by the tissue and released slowly for days.

Amino acid and peptide hydroxamates and several related derivatives have frequently been used as metalloproteinase inhibitors. In cases in which the 3-dimensional structures of the metalloproteinases have been solved, with inhibitors bound, the hydroxamate oxygens chelate the catalytic zinc, thus displacing the active-site water and blocking enzymatic activity.25–27 Adding L-tryptophan hydroxamate26 to the perfusion medium produced a gradual and sustained depression in outflow facility over several days after treatment (Fig. 3B, solid squares). Removal of the inhibitor (not shown) or addition of IL-1α after approximately 100 hours (arrow) reversed the outflow facility reduction, resulting in percentage Co at approximately pretreatment levels within a few days (solid circles, Fig. 3B).

Several related synthetic metalloproteinase inhibitors produced similar declines in outflow. A mercapto-peptide collagenase inhibitor, HS-CH₂-CH₂-CH(CH₃)₂CO-Phe-Ala-NH₃ (300 µM), produced a slow, sustained decline in outflow facility. Perfusion with 5-hydroxybenzoyl-Ala-Phe (50 µM) had

### Table 1. Metalloproteinase Inhibition Kinetics and Outflow Inhibition Concentrations

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Stromelysin (IC₅₀)</th>
<th>Gelatinase A (IC₅₀)</th>
<th>Gelatinase B (IC₅₀)</th>
<th>Inhibition of Outflow</th>
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<td>TIMP-2* (nM)</td>
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<td>5</td>
<td>5</td>
<td>50</td>
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<td>Minocycline (µM)</td>
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<td>95.5</td>
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<tr>
<td>Propeptide (µM)</td>
<td>173</td>
<td>103.7</td>
<td>115.5</td>
<td>200</td>
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</table>

TIMP, tissue inhibitor of metalloproteinase; IC₅₀, 50% inhibitory concentration.

* TIMP-2 inhibition values for the metalloproteinases were taken from the literature.
a more rapid outflow-depressing effect. A small peptide, Ac-RCGVP-NH₂, taken from the highly conserved metalloproteinase propeptide sequence, has also been shown to inhibit stromelysin. We found that this peptide depressed outflow facility effectively and when it was removed from the perfusion medium (arrow), outflow returned to pretreatment levels (Fig. 3C).

**Kinetic Analysis of Metalloproteinase Inhibitors**

To correlate the concentrations at which these compounds reduced outflow facility and inhibited metalloproteinase activity, we evaluated their inhibition of purified human stromelysin, gelatinase A, and gelatinase B, using standard enzyme kinetic analysis with their protein substrates. Inhibitor concentration curves for these enzymes' activities were used to determine IC₅₀ values for each inhibitor (Table 1). The activity curves obtained for increasing concentrations of the tryptophan hydroxamate with stromelysin, gelatinase A, and gelatinase B, respectively, are shown (Figs. 4A, 4B, 4C). Very similar curves were obtained with the other inhibitors (not shown). In parallel analysis, the Kᵣ values and mode of inhibition were determined for each enzyme-inhibitor combination (not shown). In addition, IC₅₀ and Kᵣ values were determined for these inhibitors and enzymes using the fluorescence quench-release peptide substrates NFF2 and NFF3 in continuous assays (not shown). Enzymatic inhibition and outflow facility reduction occurred in approximately the same ranges with these inhibitors.

**Trabecular Cell Viability and Perfusion Culture**

To evaluate trabecular cell viability after perfusion culture, live-dead staining and confocal microscopy of flatmounted anterior segment explants was used (Fig. 5). A fresh explant,
11 hours after death (Fig. 5A), is compared with an explant after 1 week in stationary culture (Fig. 5B). In each case, several cells from a field of several hundred exhibited insufficient membrane integrity to exclude the diethidium (dead red) nuclear-DNA stain. These same cells were also sufficiently permeable that the live stain, calcein AM, (green) was not retained, and its fluorescence was not activated within the cells by endogenous esterases. Treatment with saponin (Fig. 5C) or methanol (not shown) permeabilized the plasma membranes and allowed all the cells' nuclei to be identified. Two anterior segment explants that were in stationary culture for 1 week followed by perfusion culture for 1 week are also shown (Figs. 5D, 5E). These explants were chosen as examples of the range from good cellular viability (Fig. 5D) to relatively bad cellular viability (Fig. 5E) in the eyes that we used. In the latter case, 12% to 15% of the cells were not fully viable after the complete culture-perfusion protocol. In several cases we compared preculture and postculture eyes. When one of a pair of eyes stained immediately, and the other stained after perfusion, the ratio of dead to live cells was similar. An explant that was in stationary culture for 7 days, then in perfusion culture without treatment for 5 days, then in perfusion culture with IL-1α for 3 days is shown in Figure 5F. In this case, approximately 10% of the cells were dead, or at least their plasma membranes were somewhat compromised. An explant cultured in stationary culture for 7 days, then in perfusion culture for 6 days, then in perfusion with TIMP-2 for 3 days is shown in Figure 5G.

Also, note the band of red staining of the extracellular matrix in Figure 5A, where the diethidium penetrated between trabecular cells and bound to some component of the trabecular extracellular matrix. This can be seen as a soft haze of red staining in the permeabilized explant (Fig. 5C) and seems to be more pronounced when the optical section obtained is near the juxtacanalicular region. Because the trabecular meshwork is thicker than the maximum optical sectioning depth of this instrument (40-50 μm), the juxtacanalicular region and Schlemm's canal are normally beyond the zone of clear resolution, and the extracellular matrix staining is not in focus but is often visible.

**DISCUSSION**

These data support our working hypothesis that the trabecular outflow resistance is normally regulated, at least in part, through ongoing ECM turnover through endogenous matrix metalloproteinases. It could be argued that the outflow facility increases, produced by adding or inducing the metalloproteinases, created new flow pathways or other artifacts. However, because the inhibition of endogenous trabecular metalloproteinase activity reduced outflow facility, then normal outflow maintenance must be dependent on the ongoing activity of these enzymes, at least in the human model. It is difficult to argue that the wide array of inhibitors from the several different classes of inhibitors that we used would all act through some other molecular mechanism. This is reinforced by the reversal of the outflow reduction by removal of the inhibitors, the blockage of the IL-1α effect by TIMP-2, and the ability of IL-1α to overcome the inhibition by L-tryptophan hydroxamate. Although these results do not prove that glaucoma is normally caused by reduced trabecular ECM turnover, this is a possible interpretation of our results. Of course, extrapolation of our results from a human culture model to the in vivo human eye requires considerable caution.

A recent analysis of the morphology and cellularity of human trabecular meshwork after prolonged perfusion culture64 is in agreement with the present physiologic observation that all our effects were reversible (except minocycline, which was retained by the tissue). In that study,64 explants that were maintained in stationary culture for 1 week exhibited excellent morphology, ultrastructure, ECM, and cellularity, agreeing with our previous observation.53 Perfused explants, with flow rates higher than 1 μl/min also maintained good to excellent morphology, whether constant perfusion pressure or constant flow rate was used.64 By using a stationary preculture period of 1 week64 before beginning perfusion culture, our outflow facilities were near physiologic. The normal human trabecular outflow resistance provides a 6 mm Hg to 7 mm Hg pressure decrease from the anterior chamber to the episcleral venous drainage, and the normal or glaucomatous human daytime aqueous flow rate is 2.75 ± 0.63 μl/min.33,54 Using confocal microscopy and live-dead staining, we observed between 0% and 15% dead or at least membrane-compromised trabecular cells after 1 to 2 weeks in perfusion culture, in complete agreement with a detailed earlier report.64 Although we have not conducted a detailed analysis to verify this, it seems that the number of dead-staining cells is similar in pairs of eyes when one eye is stained immediately and the other is cultured; suggesting that cellular viability may be related to postmortem, preculture effects. A significant portion, approximately 25% of the eyes we obtained, did not stabilize to acceptable flow rates in the perfusion system and showed considerable numbers of dead cells when analyzed after culture, also in agreement with previous reports.62,64

Although minocycline produced a dramatic reduction in outflow in our study, other tetracycline derivatives have produced ocular hypotension in rabbits in vivo.74 However, in that study, the effect was on aqueous humor inflow, that is, diminished aqueous production by the ciliary body, rather than on outflow. The final aqueous humor concentrations achieved in that study and with minocycline, when used clinically as an ocular antibiotic, were far below the threshold dose that produced outflow suppression in our study (≥40 μM).

Aqueous inflow is relatively insensitive to pressure as a feedback mechanism.32-34 The 50% to 90% reductions in outflow that we observed would produce elevations in intraocular pressure, which if sustained would cause subsequent glaucomatous optic nerve head damage in vivo. Recently, elevated TIMP levels have been reported in the aqueous humor of patients with open-angle glaucoma, but not with other forms.75 Also in support of our working hypothesis, we have recently shown that a common laser treatment, used clinically to ameliorate the elevated intraocular pressure observed in open-angle glaucoma, induces dramatic and sustained metalloproteinase expression, specifically in the juxtacanalicular meshwork, the putative site of the outflow resistance.49-50

The potential of modulating aqueous outflow by manipulating trabecular matrix metalloproteinase activity has significant clinical implications. Although primary open-angle glaucoma is probably caused by a variety of genetic and environmental factors, it may well be possible to ameliorate
intraocular pressure elevations in at least a portion of glaucoma by some variation of this general approach.

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References


