Cultured Human Trabecular Meshwork Cells Express Functional Growth Factor Receptors

Robert J. Wordinger, Abbot F. Clark, Rajnee Agarwal, Wendi Lambert, Loretta McNatt, Steven E. Wilson, Z. Qu, and Bernard K.-K. Fung

PURPOSE. To compare the mRNA expression of growth factor receptors in cultured human trabecular meshwork (HTM) cells with ex vivo HTM tissues and to determine whether HTM cells generate a physiologic response after exposure to exogenous growth factors.

METHODS. The reverse transcription-polymerase chain reaction (RT-PCR) method was used to detect the expression of various growth factor receptor mRNAs using early passaged, cultured HTM cells from donors of several ages. RT-PCR on ex vivo HTM tissues from healthy donors and donors with glaucoma were also used to compare and contrast mRNA expression with cell culture results. After the exogenously administered growth factors, cell proliferation and extracellular acidification rate studies were used to measure the functional responses of HTM cells to growth factors.

RESULTS. Amplification products of the expected size for 15 growth factor receptors were detected in cultured HTM cells and in ex vivo HTM tissues. The administration of exogenous growth factors showed that (a) hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulinlike growth factor (IGF)-1, tumor necrosis factor (TNF) α, platelet-derived growth factor (PDGF)-AA, PDGF-BB, PDGF-AB, and basic fibroblast growth factor (FGF-2) stimulated cell proliferation, whereas FGF-1 (acidic), transforming growth factor (TGF) α, interleukin (IL)-1α, nerve growth factor (NGF), and FGF-7 (keratinocyte growth factor [KGF]) had no significant influence on cell proliferation; (b) TGF-β isoforms significantly inhibited EGF-stimulated trabecular meshwork cell proliferation; and (c) FGF-1 (acidic), TGF-α, EGF, IL-1α, IL-1β, HGF, TNF-α, PDGF-AA, and IGF-1 significantly stimulated extracellular acidification, whereas FGF-2 (basic), FGF-7 (KGF), TGF-β1, -β2, and NGF had no significant influence on extracellular acidification.

CONCLUSIONS. These studies show that mRNA for numerous growth factor receptors can be detected in cultured HTM cells and in ex vivo HTM tissues. They also show that many of the receptors are functional, because exogenous growth factor administration elicits a physiologic response. In vivo, these receptors may be activated by growth factors present within the aqueous humor (aquecrine) or by growth factors synthesized and released locally by trabecular meshwork cells themselves (autocrine). Specific growth factors acting through high-affinity receptors may be involved in maintaining the normal microenvironment of the HTM and also may be involved in the pathogenesis of primary open-angle glaucoma. (Invest Ophthalmol Vis Sci. 1998;39:1575-1589)

Glaucoma is a leading cause of blindness. Projected estimates of all forms of glaucoma indicate that as many as 66.8 million people may be affected worldwide by the year 2000, with 6.7 million people having bilateral blindness. In the United States, as many as 2.5 million people may have the disease. One form of glaucoma is adult-onset primary open-angle glaucoma (POAG), which involves optic neuropathy accompanied by characteristic visual field defects and is often associated with elevated intraocular pressure as a result of an inhibition of aqueous humor outflow through the trabecular meshwork. The pathophysiology of the human trabecular meshwork (HTM) in POAG has been characterized by an increase in extracellular matrix components and a decrease in the number of trabecular meshwork cells. It is, thus, probable that a defect in the structure, function, or number of HTM cells influences the pathogenesis of POAG. Studies that identify cellular control mechanisms acting within the HTM are critical to our understanding of POAG and may identify unique treatment modalities.

Polypeptide growth factors are critical molecules that control normal cell functions such as proliferation, motility, differentiation, phagocytosis, and extracellular matrix synthesis and degradation. It is clear now that not only are the actions of an individual growth factor diverse, but also the range of cells that are known to respond to specific growth factors is much wider than originally anticipated. The potential for growth factors to function through paracrine mechanisms within the HTM is...
significant, because the aqueous humor is known to contain growth factors.6,7 In addition, growth factors may act locally through autocrine mechanisms because HTM cells express mRNA for several growth factors, including basic fibroblast growth factor (FGF)-2, epidermal growth factor (EGF), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), transforming growth factor (TGF) β isoforms 1 to 3 (TGF-β1, TGF-β2, and TGF-β3), and TGF-α.8,9

Growth factors that are present either in the aqueous humor or that are produced locally by trabecular meshwork cells, must mediate their pleiotropic actions by binding to and then activating specific high-affinity cell-surface receptors. Growth factor binding is known to activate a cascade of intracellular pathways, including phospholipid metabolism, arachidonate metabolism, protein phosphorylation, calcium mobilization and transport, and transcriptional regulation.10 To understand fully the role that growth factors play in the microenvironment of the normal HTM and the HTM in POAG, it is first necessary to study the expression of specific, high-affinity growth factor receptors by HTM cells and to determine whether the expressed receptors can respond to the ligand and elicit a response. There have been a few reports on the presence of growth factor receptors in porcine trabecular meshwork cells.11-13 To date, there has not been a comprehensive study detailing which growth factor receptors are expressed in the HTM.

The objectives of this study were to use the reverse transcription-polymerase chain reaction (RT-PCR) to determine which growth factor receptors are expressed by cultured HTM cells, to determine whether ex vivo HTM tissues express similar growth factor receptor mRNA patterns, and to determine whether HTM cells express functional receptors capable of responding to the exogenous administration of specific growth factors.

METHODS

Trabecular Meshwork Dissection

The eyes of human donors were obtained from regional eye banks within 24 hours of death. The eyes were bisected equatorially, and the lens, iris, and ciliary body were removed from the anterior segment. The trabecular meshwork from each eye was obtained by making parallel cuts anterior to the scleral spur and posterior to Schwalbe’s line. Normal HTM samples were obtained from 75-, 79-, and 79-year-old donors, and glaucoma samples were obtained from 77-, 79-, and 79-year-old donors. Total RNA was obtained from the trabecular meshwork samples from each pair of donated eyes using an isolation kit (Micro RNA Isolation Kit, Stratagene, La Jolla, CA). Subsequently, cDNA was synthesized as described below.

Human Trabecular Meshwork Cell Culture

Early passaged, well-characterized, normal HTM cell lines from donors aged 6 days, 48 days, 6 months, 2 years, 18 years, 54 years, and 80 years were used in studying the mRNA expression of growth factor receptors. The cell line from the 54-year-old (HTM-10) was used for the cell proliferation and extracellular acidification rate studies. This cell line has been well characterized previously.14,15 The trabecular meshwork cells were grown until confluent in Ham’s F-10 Media (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT), 2 mM L-glutamine (0.292 mg/ml), penicillin (100 units/ml), and streptomycin (0.1 mg/ml; Life Technologies, Grand Island, NY). Cells were incubated at 37°C in 7% CO2/93% air. The medium was changed every 3 days. HTM cell lines were propagated as described by Steely et al.14 Briefly, number 3 Cytodex beads (Sigma, St. Louis, MO) in a 2% suspension in sterile phosphate-buffered saline were added to confluent monolayers. Seven days later, the monolayer was gently washed using a stream of culture media, and dissodged cell-covered beads were transferred to new plates. The cells that remained on the original plate were allowed to regrow into an additional monolayer. It has been reported that Cytodex beads allow a longer retention of trabecular cell morphology than methods using trypsin.14

Total Cellular RNA Extraction and cDNA Synthesis

Total cellular RNA was prepared using a kit (RNAzol B kit; Biotex Laboratories, Houston, TX). After ethanol precipitation, the RNA was resuspended in 20 μl water and was stored at −80°C. First-strand cDNA synthesis was prepared from total cellular and trabecular meshwork tissue RNA. Initially, to reduce secondary structure, RNA (20 μg) and random primers (0.75 μg; Promega, Madison, WI) were combined and incubated at 85°C for 3 minutes. The following were then added to the reaction tube: 80 units RNAsin (Promega), 40 units avian myeloblastosis virus reverse transcriptase (Promega), 0.625 mM each deoxyribonucleotide, 50 mM Tris HCl, 75 mM potassium chloride, 10 mM dithiothreitol, and 3 mM magnesium chloride. The reaction tube was incubated at 42°C for 30 minutes followed by an incubation at 94°C for 2 minutes. The cDNA was stored at −20°C until used for PCR. A PCR reaction for β-actin (see below) was performed on each cDNA sample to ensure adequate synthesis and the absence of genomic DNA.

Primer Design

A computer program (Oligos 4.0 Software Program; National Biosciences, Plymouth, MN) was used to design PCR primers that had optimal annealing temperatures and that would amplify at similar temperatures and magnesium concentrations. Table 1 lists the upstream primer, downstream primer, annealing temperature, and expected PCR product size (bp) for the human growth factor receptor primer pairs used in this study. Human primers were designed from their GenBank sequence. Each individual primer pair sequence was submitted using the Basic Local Alignment Search Tool,16 (available online from the National Center for Biotechnology Information, Bethesda, MD) to verify that primers would not hybridize to any other known nuclear acid sequences under the conditions used. All primer pairs were designed so that the amplification of potentially contaminating genomic DNA sequences would produce PCR products that would be substantially larger than expected mRNA PCR products because intron sequences that were excised during RNA processing would be included in genomic DNA. To ensure adequate cDNA synthesis, a primer pair for β-actin was designed and was used as an internal control. The upstream primer (5’-3’) was AGGCGCAACCAGAGAGATGACC, and the downstream primer (5’-3’) was GAGTGTCAGGGCGCGACGAC. This primer pair had an optimal annealing temperature of 55°C and yielded a PCR product of 350 bp after undergoing electrophoresis.

Downloaded From: http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933208/ on 06/23/2017
Polymerase Chain Reaction, Horizontal Gel Electrophoresis, and Sequencing Polymerase Chain Reaction Products

Details of the PCR procedure used have been published previously. All samples were amplified with a primer pair that was specific for each growth factor receptor using a master mix containing all the components in the PCR reaction, except the target template cDNA or water as a negative control. All PCR reactions were prepared using the Taq Start Antibody Hot Start method (Clontech Laboratories, Palo Alto, CA) in which the target and PCR master mix solution were brought to 94°C, followed by 92°C for 2 minutes, then 40 cycling (PTC-100; MJ Research, Watertown, MA) was performed in this manner for a period of 2 weeks, with fresh medium and growth factor added two times a week. The cells were harvested using 0.05% trypsin/0.53 mM EDTA (Life Technologies) and by sequencing with Sequenase 2.0 (United States Biochemical, Cleveland, OH). The number of HTM cells in the growth factor-treated wells was compared with untreated control wells, and the remaining three wells were incubated overnight to allow the cells to attach to the plate. The high-serum medium was removed, and cells were washed with Ham’s F-10 medium with 0.5% FBS. Incubation was continued using 2.0 ml/well low-serum medium. Three wells per plate received medium containing the specified concentration of growth factor, and the remaining three wells were incubated with low-serum medium only and served as control, unstimulated wells. Cells were maintained this manner for a period of 2 weeks, with fresh medium and growth factor added two times a week. The cells were harvested using 0.05% trypsin/0.53 mM EDTA (Life Technologies) and were counted on a Coulter Counter (Coulter, Hialeah, FL). The number of HTM cells in the growth factor-treated wells was compared with untreated control wells, and the results were expressed as a percentage of untreated control cells. Statistical comparisons were performed using a one-tailed Student’s t-test with unequal variances, and P ≤ 0.05 was considered significant. Each growth factor was tested at a single concentration in multiple experiments.
corresponding number of untreated, control cells. A list of growth factors were purchased from R & D Systems (Minneapolis, MN) and used at known physiological concentrations. In an attempt to show that TGF-β isoforms may inhibit HTM cell proliferation, the number of HTM cells in the growth factor-treated wells was compared with untreated control wells, and the results were expressed as a percentage of untreated control cells. Statistical comparisons were performed using one-tailed t-test with unequal variances, and \( P \leq 0.05 \) was considered significant. Each growth factor was tested at a single concentration in multiple experiments, providing a total of 6 to 12 wells of treated cells and the corresponding number of untreated, control cells. A list of growth factors and the physiologic concentrations used in HTM cell proliferation assays is shown in Table 2. With the exception of TGF-β2 (Genzyme Diagnostic, Cambridge, MA) and double-chained HGF (Genetech, San Francisco, CA), all growth factors were purchased from R & D Systems (Minneapolis, MN) and used at known physiological concentrations.

### Negative Effect of Transforming Growth Factor β Isoforms on Epidermal Growth Factor–Stimulated Human Trabecular Meshwork Cell Proliferation

In an attempt to show that TGF-β isoforms may inhibit HTM proliferation, HTM cells were cultured for 2 weeks in Ham's F-10 medium with: 0.5% FBS in the presence of EGF (60 ng/ml) alone; EGF (60 ng/ml) in combination with either TGF-β1 (0.6 ng/ml), TGF-β2 (4.0 ng/ml), or TGF-β3 (0.6 ng/ml); or each of the three TGF-β isoforms alone. At the end of the 2-week exposure time, cells were harvested and counted as described earlier. The number of HTM cells in the growth factor-treated wells was compared with untreated control wells, and the results were expressed as a percentage of untreated control cells. Statistical comparisons were performed using one-tailed Student's t-test with unequal variances, and \( P \leq 0.05 \) was considered significant. Each growth factor was tested at a single concentration in multiple experiments, providing a total of three wells of treated cells and the corresponding number of untreated, control cells.

### Trabecular Meshwork Cell Acidification Assay

The increase in cellular metabolism of cultured HTM cells in response to exogenous growth factors was measured using a microphysiometer (Cytosensor Microphysiometer; Molecular Devices, Sunnyvale, CA). The instrument contains a parallel set of eight light-addressable potentiometric sensors that detect small changes in the extracellular pH of cells in a 1.5-μl chamber. A computer controls the pumps for the delivery of medium to the chambers, the switching valves, the temperature of the sensor chambers, and the acquisition of data.

For our microphysiometric measurements, we used an HTM cell line (HTM-10) that previously has been well characterized and that was obtained from a 54-year-old donor.\(^{14,15}\) Twelve hours before the Cytosensor measurement, the culture medium was replaced with Ham's F-10 lacking FBS. On the day of the measurement, the capsule cups containing cultured HTM cells were prepared and transferred to the sensor chamber, which was maintained at 37°C. A running buffer (low-buffered Ham's F-12; Life Technologies) containing 1.0 mg/ml bovine serum albumin was pumped at a constant flow rate (100 μl/min) from the primary fluid reservoir into the chamber at an on-off cycle of 60 and 30 seconds, respectively, to maintain cell viability. During the pump-off period, the acidification rate of the extracellular medium in the sensor chamber was determined. Data were collected over a period of 6 to 10 hours.

Growth factors were prepared as concentrated stock solutions and were stored at ~80°C. Different concentrations of the test growth factor were then freshly prepared using serial dilution with running buffer. Each agent was applied to the cells in the sensor chamber for four on-off cycles over a period of 6 minutes using a solenoid valve to regulate the flow of medium from a secondary reservoir. Each concentration of the growth factor was repeated at least two or three times in different experiments. Because the basal acidification rate in each chamber depends on the number of cells near the active

---

**Table 2. Proliferation Response of Human Trabecular Meshwork Cells to Growth Factors**

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Dose (ng/ml)</th>
<th>n</th>
<th>Proliferation as Percentage of Control (± SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Significant HTM cell response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>60.0</td>
<td>12</td>
<td>197.30 ± 63.6</td>
<td>0.01</td>
</tr>
<tr>
<td>FGF-1 (acidic)</td>
<td>6.0</td>
<td>6</td>
<td>118.80 ± 17.0</td>
<td>0.05</td>
</tr>
<tr>
<td>FGF-2 (basic)</td>
<td>2.4</td>
<td>9</td>
<td>205.30 ± 10.3</td>
<td>0.01</td>
</tr>
<tr>
<td>HGF</td>
<td>5.0</td>
<td>9</td>
<td>227.30 ± 46.9</td>
<td>0.01</td>
</tr>
<tr>
<td>IGF-1</td>
<td>60.0</td>
<td>9</td>
<td>135.30 ± 25.0</td>
<td>0.01</td>
</tr>
<tr>
<td>PDGFA</td>
<td>100.0</td>
<td>6</td>
<td>184.80 ± 57.1</td>
<td>0.01</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>60.0</td>
<td>6</td>
<td>193.10 ± 48.8</td>
<td>0.01</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.4</td>
<td>12</td>
<td>175.00 ± 79.3</td>
<td>0.01</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>0.6</td>
<td>6</td>
<td>74.20 ± 14.3</td>
<td>0.01</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>4.0</td>
<td>6</td>
<td>66.70 ± 13.9</td>
<td>0.01</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>0.6</td>
<td>6</td>
<td>58.30 ± 11.0</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Nonsignificant HTM cell response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>0.1</td>
<td>9</td>
<td>113.30 ± 32.6</td>
<td>0.27</td>
</tr>
<tr>
<td>KGF</td>
<td>5.0</td>
<td>6</td>
<td>90.50 ± 7.6</td>
<td>0.35</td>
</tr>
<tr>
<td>NGF</td>
<td>20.0</td>
<td>6</td>
<td>110.90 ± 20.8</td>
<td>0.24</td>
</tr>
<tr>
<td>PDGF-AB</td>
<td>100.0</td>
<td>6</td>
<td>117.00 ± 61.8</td>
<td>0.27</td>
</tr>
<tr>
<td>TGF-α</td>
<td>8.0</td>
<td>6</td>
<td>109.60 ± 16.5</td>
<td>0.28</td>
</tr>
<tr>
<td>VEGF</td>
<td>40.0</td>
<td>6</td>
<td>108.40 ± 24.0</td>
<td>0.23</td>
</tr>
</tbody>
</table>

HTM, human trabecular meshwork; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; TNF, tumor necrosis factor; TGF, transforming growth factor; IL, interleukin; KGF, keratinocyte growth factor; NGF, nerve growth factor; VEGF, vascular endothelial growth factor.
site of the sensor, the responses to growth factors were routinely normalized to the basal level for comparing different samples. The total response was determined by integrating the increase in the normalized acidification rate over the basal level.

### RESULTS

#### Control Polymerase Chain Reactions

Figures 1, 2, and 3 show that each of the cDNA samples obtained from cultured HTM cell lines and ex vivo trabecular meshwork tissues expressed detectable actin PCR amplification products of the expected size (350 bp) without evidence of a 790-bp genomic amplification product (data not shown). These results show the high quality of the cDNA used for PCR detection of mRNA expression. In addition, during PCR amplification reactions all negative control reactions lacked amplification products, demonstrating that the PCR method and reagents were used and yield specific amplified products only when a cDNA source was included.

#### Cultured Human Trabecular Meshwork Cells Express mRNA for Numerous Growth Factor Receptors

HTM cells and ex vivo HTM tissue express mRNA for at least 15 growth factor receptors. We have included only representative examples of PCR reaction products. Figure 1 shows that mRNA for growth factor receptors are expressed by HTM cell lines from donors of various ages. The following growth factor receptors were detected in trabecular meshwork cells from donors ranging in age from 6 months to 80 years: EGF receptor (1139 bp), FGF receptor-1 (880 bp), HGF receptor (336 bp), TGF-β receptor-1 (242 bp), and TGF-β receptor-2 (543 bp). In addition to the PCR products of receptors shown in Figure 1, we also have found mRNA expression for FGF receptor-2 (beak) (199 bp), FGF receptor-4 (240 bp), KGF receptor (172 bp), platelet-derived growth factor (PDGF) receptor-α (756 bp), PDGF receptor-β (233 bp), TGF-β receptor-III (422 bp), insulin-like growth factor (IGF) receptor-1 (498 bp), IGF receptor-2 (526 bp), interleukin (IL)-1 receptor (379 bp), and tumor necrosis factor (TNF) receptor-1 (55 kDa; 363 bp) in these same cell lines (data not shown). Nucleic acid sequencing of each product of the expected size showed conclusively that the amplified products were derived from mRNA for the specific growth factor receptor.

#### Ex Vivo Human Trabecular Meshwork Tissues Express mRNA for Numerous Growth Factor Receptors

Figures 2 and 3 show that mRNA for growth factor receptors is expressed by ex vivo HTM tissues obtained from healthy donors and donors with glaucoma. Representative examples of mRNA expression in both tissue types are included. Figure 2 shows FGF receptor-1 (880 bp), IL-1 receptor (379 bp), and TGF-β receptor-2 (543 bp), and in Figure 3 PDGF receptor-α (756 bp), PDGF receptor-β (233 bp), and TNF receptor-1 (55 kDa; 363 bp) are expressed in the same six donor tissues. In addition to the receptors shown in Figures 2 and 3 we have found mRNA expression for FGF receptor-2 (beak) (199 bp), FGF receptor-4 (240 bp), KGF receptor (172 bp), EGF receptor (1139 bp), HGF receptor (336 bp), TGF-β receptor-1 (242 bp), TGF-β receptor-III (422 bp), IGF receptor-1 (498 bp), and IGF receptor-2 (526 bp). Nucleic acid sequencing of each product of the expected size showed conclusively that the amplified products were derived from mRNA for the specific growth factor receptor.

#### Cultured Human Trabecular Meshwork Cells Proliferate in Response to Exogenous Growth Factors

Proliferation results after the exogenous administration of growth factors to the cell line from the 54-year-old donor (HTM-10) are summarized in Table 2. Significant proliferation responses (P ≤ 0.05) were obtained after the exogenous administration of physiologically relevant concentrations of EGF, FGF-1, FGF-2, HGF, IGF-1, PDGF (AB, and BB), and TNF-α. Nonsignificant proliferation responses were obtained after the exogenous administration of physiologically relevant concentrations of IL-1α, KGF, nerve growth factor (NGF), PDGF-α, TGF-α, and vascular endothelial growth factor. Mitogenic responses to specific growth factors by HTM cells strongly suggest that the corresponding receptor mRNAs are translated into functional proteins in these cells.

#### Transforming Growth Factor β Isoforms Inhibit Epidermal Growth Factor–Stimulated Cell Proliferation

Proliferation results after TGF-β treatment of EGF-stimulated HTM cells are summarized in Table 3. A negative growth effect of each of the three TGF-β isoforms on HTM cells (Table 2) was confirmed. The treatment of HTM cells with 60.0 ng/ml EGF significantly stimulated cell proliferation, which is consistent with our previous results. The administration of each of the three TGF-β isoforms significantly inhibited the proliferative response seen with EGF alone. These results indicate that functional TGF-β receptor proteins (TGF-β receptor-1 and TGF-β receptor-2) are present in HTM cells and act to inhibit cell proliferation.

#### Exogenous Growth Factor Administration Causes Extracellular Acidification by Cultured Human Trabecular Meshwork Cells

Changes in extracellular acidification by HTM cells were measured continuously after exposure to various concentrations of growth factors using silicon-based microphysiometry technology. However, because metabolic responses to receptor stimulation are best observed in cells maintained in serum-free or low-serum media and because serum starvation often is used in bioassays for growth factors, we first needed to establish the effect of serum starvation on cultured HTM cells. HTM cells showed stable metabolic rates (uninduced) in serum-free media (data not shown). These results indicate that serum starvation did not create a pathologic condition that resulted in irrelevant responses. Thus, this preliminary study showed that the extracellular acidification rate, as measured using the microphysiometer, can be used to detect the activation of specific growth factor receptors using HTM cell lines.

Extracellular acidification results after the exogenous administration of growth factors to the cell line from the 54-year-old donor (HTM-10) are summarized in Table 4. Results were reported as a percentage of the basal rate. Specifically, our
studies indicate that the exogenous administration of EGF, FGF-1, HGF, IGF-1, IL-1 (α and β), PDGF-AA, TGF-α, and TNF-α resulted in significant increases in extracellular acidification by HTM cells. Our results also showed that these increases were dose dependent. The peak response for each dose of growth factor was determined and plotted versus concentration. The resultant EC₅₀ (the effective concentration of the growth factor that elicited a 50% of maximal cell response) for each growth factor was calculated and also is included in Table 3. The EC₅₀ for the various growth factors ranged from 0.5 ng/ml (EGF,
FIGURE 2. Ethidium bromide-stained agarose gel of representative growth factor receptor polymerase chain reaction (PCR) products from cDNA samples generated from human ex vivo trabecular meshwork tissues from healthy donors and donors with glaucoma. Receptor PCR products presented include fibroblast growth factor receptor-1 (FGFR-1 (flg)), interleukin-1 receptor (IL-1R), and transforming growth factor β receptor-II (TGFβR-II). Actin was included as an internal standard. HTM, human trabecular meshwork; GTM, glaucomatous trabecular meshwork.

FGF-1, and IL-1β) to 30 ng/ml (HGF) and are representative of physiological levels of these growth factors.

In addition to the EC_{50} for the respective growth factor, a time-course pattern of acidification can be obtained. Several different extracellular acidification stimulation patterns were observed. Representative examples are shown in Figures 4 to 7. The first type of response is illustrated by the addition of TGF-α (Fig. 4). The addition of TGF-α resulted in a peak response in 5 to 10 minutes, with the peak lasting for an additional 10 minutes. This was then followed by a gradual decline to basal levels. This time-course pattern was the predominant response pattern, inasmuch as it was also observed with the addition of HGF, EGF, FGF-1 (acidic), IL-1α, and IL-1β (data not shown). The second type of response pattern that we observed is illustrated by the addition of IGF-1 (Fig. 5) and results in a double peak response. One peak was seen at approximately 10 minutes, followed by a decline and then a second greater response peak 15 to 20 minutes later. This time-course pattern was seen only with the administration of IGF-1. The third type of response is illustrated by the addition of PDGF-AA (Fig. 6), which results in a rapid response peak within 5 minutes and then a sharp decline that then levels off.
FIGURE 3. Ethidium bromide-stained agarose gel of representative growth factor receptor polymerase chain reaction (PCR) products from cDNA samples generated from human ex vivo trabecular meshwork tissues from healthy donors and donors with glaucoma. Receptor PCR products presented include platelet-derived growth factor receptor (PDGFR)-α, PDGFR-β, and tumor necrosis factor receptor (TNFR). Actin was included as an internal standard. HTM, human trabecular meshwork; GTM, glaucomatous trabecular meshwork.

and declines more gradually. This time-course pattern was also seen with the addition of TNF-α.

As can be seen in Table 4, several growth factors failed to stimulate extracellular acidification. These included FGF-2 (basic), KGF, NGF, and all TGF-β isoforms (namely, -β1, -β2, and -β3). A typical time-course response pattern for these growth factors is illustrated for TGF-β2 (Fig. 7) and shows the lack of extracellular acidification with exposure to various concentrations of TGF-β2. Table 5 compares the cell proliferation and extracellular acidification results. The cell responses fell into the following categories: growth factors that stimulated cell proliferation and extracellular acidification; growth factors that stimulated either cell proliferation or extracellular acidification; and growth factors that stimulated neither cell proliferation or extracellular acidification.

**DISCUSSION**

This study examined the expression and function of multiple growth factor receptors within the HTM. There were three significant findings in the study. First, using RT-PCR technology, we have showed that cultured HTM cells express mRNAs for 15 different growth factor receptors. Second, we have showed a similar mRNA expression pattern for growth factor receptors within fresh ex vivo trabecular meshwork tissues. Third, the exogenous administration of growth factors to cultured trabecular meshwork cells stimulated cell proliferation and extracellular acidification, demonstrating the presence of translated, functional receptor proteins. As a corollary to the latter finding, we also have showed that isoforms of TGF-β inhibited HTM cell proliferation. 
TABLE 3. Negative Effects of TGF-β1, TGF-β2, and TGF-β3 on EGF-Stimulated Human Trabecular Meshwork Cell Proliferation

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Dose (ng/ml)</th>
<th>Proliferation as Percentage of Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>0.6</td>
<td>84.5 ± 12.0</td>
<td>0.01</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>4.0</td>
<td>77.7 ± 5.3</td>
<td>0.01</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>0.6</td>
<td>54.2 ± 13.6</td>
<td>0.01</td>
</tr>
<tr>
<td>EGF</td>
<td>60.0</td>
<td>225.3 ± 22.7</td>
<td>0.01</td>
</tr>
<tr>
<td>EGF + TGF-β1</td>
<td>60.0 + 0.6</td>
<td>89.7 ± 6.8</td>
<td>0.01</td>
</tr>
<tr>
<td>EGF + TGF-β2</td>
<td>60.0 + 4.0</td>
<td>87.7 ± 20.7</td>
<td>0.01</td>
</tr>
<tr>
<td>EGF + TGF-β3</td>
<td>60.0 + 0.6</td>
<td>86.5 ± 11.6</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Values are means ± SD.

Messenger RNAs for members of several growth factor receptor families were detected using RT-PCR in early pas- saged HTM cell lines from donors of various ages (namely, 6 days to 80 years). To our knowledge, this is the first report that shows the mRNA expression of several of these growth factor receptors in the HTM. The identical growth factor receptor mRNA pattern also was detected using RT-PCR in ex vivo HTM tissues. These results prove that the expression of growth factor receptor mRNAs is not an artifact of tissue culture conditions. We are not aware of other studies reporting mRNA expression of growth factor receptors by cells within ex vivo HTM tissues. Because numerous growth factor receptor mRNAs are expressed in trabecular meshwork tissues, a potentially large number of growth factors may be biologically active and may function within this tissue.

Having established that HTM cells express mRNA for several growth factor receptors, we wished to extend these find- ings by determining whether the expression of an individual growth factor receptor mRNA was translated into a functional protein. This is important because the translation of a specific mRNA may be repressed or altered using post-transcriptional regulatory mechanisms. In addition, functional data of this type give us a better understanding as to which growth factors may potentially be involved in the pathophysiology of this tissue. To show the presence of functional growth factor receptors, we administered exogenous growth factors to cul- tured HTM cells and measured cell proliferation and extracel- lular acidification responses. The concentration of each growth factor used in the study was within the physiologic range and had been previously shown to activate other cell types.

Cell proliferation within the HTM is an important physio- logic response to consider because of previous reports that indicated that the HTM undergoes an age-related decrease in cell numbers, which is accelerated in POAG. Because of these previous studies, it has been suggested that trabecular meshwork cell number may play a role in the pathogenesis of glaucoma. As proposed by Kimpel and Johnson, the decrease in cell number within the trabecular meshwork may be a result of a low intrinsic cell number in the trabecular meshwork, an accelerated cell loss, or aqueous humor factors that influence cell proliferation. Because human aqueous humor contains growth factors that are known to control cell proliferation and differentiation in other cell types, it is critical to our understanding of the normal function of the trabecular meshwork to determine which growth factor receptors are involved in cell proliferation.

Previous studies have examined the role of growth factors in the proliferation of cultured HTM cells. Fauss et al. reported that FGF-2 and PDGF stimulated cell division, although the isoform of PDGF was not indicated. Their report confirmed the original observations that FGF-2 was a cell mitogen. These studies were the first to establish that FGF-2 stimulated HTM cell proliferation. Results from our present study indicate that several other growth factors including FGF-1, HGF, IGF-1, and TNF-α also are capable of stimulating the cell proliferation of cultured HTM cells. For these growth factors to stimulate cell proliferation, they must bind to and activate their respective high-affinity receptor and stimulate second messenger pathways.

Our results also show that several growth factors including IL-1α, KGF, NGF, PDGF-AA, and vascular endothelial growth factor failed to stimulate HTM cell proliferation. This is...
in agreement with previous studies that used other cell lines and porcine, bovine, and human trabecular meshwork cells. However, studies that expose cells to a particular growth factor and note a lack of cell proliferation do not prove an inhibitory effect of that growth factor. Such studies only show that the particular growth factor cannot stimulate cell prolifer-

--Figure 4--

**FIGURE 4.** Extracellular acidification rate increase after the exogenous administration of transforming growth factor (TGF) α. The peak response is obtained in 5 to 10 minutes, followed by a gradual decline to basal levels. BSA, bovine serum albumin.

---

**FIGURE 5.** Extracellular acidification rate increase after the exogenous administration of insulin-like growth factor (IGF)-1. There is an initial peak at 5 to 10 minutes, followed by a decline and then a second peak at 20 to 25 minutes. This is then followed by a gradual decline to basal levels. BSA, bovine serum albumin.
Growth Factor Receptors in the Trabecular Meshwork

To show that TGF-β downregulates/inhibits cell proliferation in HTM cells, we first stimulated cell proliferation with EGF and then treated the cell line with one of the three isoforms of TGF-β. Each TGF-β isoform downregulated EGF-stimulated cell proliferation.

The second physiologic response that we examined was the change in cellular metabolism. As reported previously, the interaction of ligands with a variety of functional receptors in living cells can be confirmed by measuring the change in extracellular pH. This measurement is based on the observation that stimulation of cell surface receptors increases the production of acidic metabolites such as carbonic acid and lactic acid in cultured cells, resulting in a transient change in extracellular acidification rate. In this study we were able to detect an increase in extracellular acidification rate in response to several growth factors in a dose-dependent manner. With the exception of FGF-2 (basic), the microphysiometric measurement correlated well with the cell proliferation assay (Table 5). The increase in extracellular acidification rate can be explained by an increase in glycolysis associated with initiation of a translation of cells from a dormant nonproliferative state to a proliferative state after exposure of the HTM cells to growth factors. Previous studies using other cell types have also reported increases in cellular glycolytic rates in response to hormones and growth factors including insulin and epidermal growth factor (EGF).

We also observed differences in the pattern of metabolic response evoked by different growth factors. For example, the most common response to a growth factor by trabecular meshwork cells was illustrated by TGF-α, which elicits an initial response in 5 to 10 minutes and then a gradual decline to baseline levels. A different response pattern was observed with IGF-1 and included an initial peak response in 10 minutes, a decline, and then a second higher peak response 15 to 20 minutes later. A third type of pattern was illustrated by PDGF-AA, which elicits a very rapid response within 5 minutes and then a decline to baseline levels. The various patterns of extracellular acidification indicates that individual growth factors, acting through high-affinity receptors, can signal HTM cells differently and elicit different response patterns. These response patterns may be correlated with different physiologic responses to a given growth factor. This is a further indication that the HTM is a extremely complex tissue that is capable of responding to a number of growth factors in a number of unique ways.

When one summarizes cell proliferation and extracellular acidification responses to individual growth factors (Table 5), it is clear that three distinct patterns exist. These include: growth factors that stimulated cell proliferation and extracellular acidification; growth factors that stimulated either cell proliferation or extracellular acidification; and growth factors that stimulated neither cell proliferation or extracellular acidification. Several growth factors elicited a dual response with an ability to stimulate extracellular acidification and cell proliferation. This would not be surprising because extracellular acidification would be associated with cell proliferation. This type of response indicates that active, functional growth factor receptors are present in the HTM for these growth factors. IL-1α, IL-1β, and TGF-α elicited extracellular acidification but failed to stimulate cell proliferation. It is possible that a different cellular...
response was activated other than cell proliferation. For example, cell motility and phagocytosis could be activated, but these responses were not measured in our study. The exogenous administration of some growth factors failed to stimulate either a cell proliferation or an extracellular acidification response. Because mRNA for these receptors was detected with PCR, an argument could be made that the expression of mRNA for these receptors is not translated into functional receptor protein. This seems unlikely for the TGF-β receptors, because Tripathi et al. have reported that porcine trabecular meshwork cells express receptors that bind TGF-β1 and TGF-β2. In addition, our current results indicate that the addition of TGF-β isoforms inhibited cell proliferation, thus demonstrating the presence of functional receptors. However, at the present time we have not performed western blot analysis or immunohistochemistry to verify whether KGF and NGF receptor proteins are present in HTM cells or HTM tissues. An alternate explanation may be that the level of expression of KGF and NGF receptors was not translated into a sufficient number of molecules to elicit a functional response. A third possibility is that functional growth factor receptors are present, but their activation does not result in either cell proliferation or in acidification but rather in a different response, which we failed to measure.

We think that it is important to consider the potential mechanisms by which growth factors are signaling HTM cells. One mechanism may be through the aqueous humor. Measurable quantities of TGF-β2, FGF-2 (basic), IGF-1, and EGF have been reported in the aqueous humor. Significantly, our present study has shown that receptors for these particular growth factors also are expressed by HTM cells. Several potential sources for growth factors within the aqueous humor can be identified. Ciliary body epithelial cells, corneal endothelial cells, and lens epithelial cells are known to produce growth factors. Wilson et al. and Weng et al. have suggested the term aquecrine (namely, modified paracrine) to describe the uniqueness of this fluid to support cellular communication among various cell populations that are bathed by aqueous humor. A second mechanism by which growth factors may be acting within the trabecular meshwork is through autocrine signaling. Previous studies using porcine trabecular meshwork cells showed that TGF-β1 and TGF-β2 are produced and secreted and that FGF-2 (basic) can be immunolocalized within this tissue. We have reported previously that HTM cells express mRNA for individual growth factors including FGF-2 (basic), EGF, HGF, KGF, TGF-β1, TGF-β2, TGF-β3, and TGF-α. Because our current results show that functional receptors for these individual growth factors also are expressed by HTM cells, the distinct possibility exists that signaling also occurs through an autocrine mechanism.

Growth factors present within the microenvironment of the HTM may play a significant role in maintaining the normal physiology of this tissue. It has been proposed also that growth factors play a role in the pathogenesis of POAG. For example, Tripathi et al. reported that aqueous humor from patients with glaucoma contained elevated levels of TGF-β2. They speculated that the elevated levels of active TGF-β2 could increase extracellular matrix protein synthesis and secretion, could reduce trabecular meshwork cellularity by inhibiting cell proliferation, or both. Our results support the latter suggestion, because we report that TGF-β isoforms inhibit HTM proliferation. Thus, as cells are lost from the HTM, TGF-β within the tissue be reduced.
TABLE 5. Summary of Cultured Human Trabecular Meshwork Cell Proliferation and Extracellular Acidification Responses to Exogenous Growth Factors

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Proliferation</th>
<th>Extracellular Acidification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Significant cell proliferation and significant extracellular acidification (+/+ +)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FGF-1 (acidic)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HGF</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IGF-1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PDGF-AB</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TNG-α</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Significant cell proliferation or significant extracellular acidification (+/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF-2 (basic)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IL-1α</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL-1β</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PDGF-AA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TGF-α</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TGF-β1*</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TGF-β2*</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TGF-β3*</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Nonsignificant cell proliferation and nonsignificant extracellular acidification (-/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KGF</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NGF</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulinlike growth factor; PDGF, platelet-derived growth factor; TNF, tumor necrosis factor; IL, interleukin; TGF, transforming growth factor; KGF, keratinocyte growth factor; NGF, nerve growth factor.

* The significant response to each TGF-β isoform was an inhibition of EGF-stimulated cell proliferation.

aqueous humor could inhibit localized cell proliferation. Activation of cell proliferation within the trabecular meshwork may be dependent on a specific combination of growth factors or on the concentration ratios between specific growth factors.7 Our results concerning TGF-β and EGF support this suggestion because the concentration of TGF-β isoforms used was sufficient to inhibit the proliferative action of EGF. It is also quite possible that growth factors activate and control other physiologic responses within the HTM, which we have not measured in the present study (namely, motility, phagocytosis, and extracellular matrix modifications). For example, Tamura and Iwamoto45 showed that PDGF enhanced the phagocytic activity of cultured HTM cells and altered actin cytoskeletal elements. In addition, Tamm et al.46 have shown that TGF-β1 induces α-smooth muscle actin expression in cultured HTM cells. Recently, Wirz et al.47 used western blot analysis and immunohistochemistry to show the presence of the IGF-I receptor in human and porcine trabecular meshwork cells. They showed further that IGF-I, but not IGF-II, increased the expression of trabecular stromelysin and gelatinase B.

A potentially important aspect of growth factor action on HTM cells has been reported by Polansky and coworkers.48,49 In the initial study the investigators showed that FGF-2 and TGF-β (isoform not identified) decreased glucocorticoid-induced myocilin expression. Myocilin is the product of the GLC1A gene, and myocilin mutations have been reported to be responsible for juvenile and a small percentage of adult-onset POAG.51 The investigators reported that each growth factor reduced the induction of myocilin by 30% individually but by 50% in combination. EGF did not seem to have an effect. Because of the potential clinical significance, this study should be verified and expanded to determine whether other growth factors also influence myocilin expression.

In conclusion, the HTM expresses numerous growth factor receptors that are capable of responding to exogenous ligands. These results emphasize the complexity of the trabecular meshwork. The presence of multiple growth factor receptors gives the trabecular meshwork the ability to respond to growth factors that either are present in the aqueous humor (aquecrine/paracrine) or are produced locally by trabecular meshwork cells (autocrine). Another level of complexity has been discovered within the trabecular meshwork since we reported that mRNAs for alternate spliced isoforms of HGF receptor, KGF receptor, and TGF-β receptor-II are expressed by HTM cells.52 Finally, there has been virtually no attempt to study second-order responses of growth factors within the trabecular meshwork. For example, we know little about what influence the action of one growth factor may have on the expression of another growth factor or growth factor receptor. Supporting the significance of this suggestion is the recent report by Li et al.,53 who showed that TGF-β1 and TGF-β2 positively regulate the expression of TGF-β1 mRNA in porcine trabecular meshwork cells. It is clear that growth factors and growth factor receptors may play a critical role in maintaining the normal function of the trabecular meshwork and may be involved in the pathophysiology of POAG. Our challenge in the future is to determine the specific role that growth factors play within the microenvironment of the trabecular meshwork and...
to determine whether the manipulation of endogenous growth factors potentially may offer new therapeutic opportunities.

Acknowledgments

The authors thank Sherry English-Wright for assistance in the cell culture and dissection of the HTM, Lawrence Oakford for help in figure presentation, and The Central Florida Lions Eye and Tissue Bank for providing the human eyes used in the study.

References


