Transforming Growth Factor-\(\beta1\) and \(-\beta2\) Positively Regulate TGF-\(\beta1\) mRNA Expression in Trabecular Cells

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Purpose. To determine whether transforming growth factor (TGF)-\(\beta1\) and \(-\beta2\) and basic fibroblast growth factor (bFGF) induce the gene expression of TGF-\(\beta1\) in the first-passage trabecular meshwork cells of the eye.

Methods. Trabecular meshwork cells were cultured from fresh porcine eyes and treated with 1 ng/ml of TGF-\(\beta1\), TGF-\(\beta2\), or bFGF for 1 hour. Cells maintained in serum-free medium were used as controls. Total cellular RNA was extracted, and the first-strand cDNA was synthesized. Multiplex polymerase chain reaction (PCR) and competitive PCR were performed on aliquots of the cDNAs by using either endogenous (glyceraldehyde-3-phosphate dehydrogenase [G3PDH]) or exogenous sequence (PCR mimic for TGF-\(\beta1\)) as internal standards, respectively. The obtained products were quantitated by laser densitometry, and statistical analysis was performed.

Results. The findings show that trabecular cells in vitro express the TGF-\(\beta1\) messenger RNA constitutively. Both the techniques of multiplex PCR and competitive PCR demonstrated that the addition of either TGF-\(\beta1\) or TGF-\(\beta2\) at a concentration present in normal aqueous humor increased the mRNA levels of TGF-\(\beta1\) by 2.82- to 3.07-fold over the controls, and these results were statistically significant \((P < 0.01)\). Basic fibroblast growth factor did not have an effect on TGF-\(\beta1\) expression \((P > 0.05)\).

Conclusions. Transforming growth factor-\(\beta1\) activates its own gene expression in trabecular cells. Considering the multifunctional property of this cytokine, which includes increased deposition of extracellular matrix material and growth inhibition of trabecular cells, a change in its concentration within the eye would have a profound effect because of this autoinductive activity.
Transforming growth factor-β2 treatment of trabecular cells also increased their expression of the TGF-β1 gene. The authors previously showed that the level of TGF-β2 in the aqueous humor of glaucomatous eyes is significantly higher than that of age-matched nonglaucomatous controls. The current finding suggests that this growth modulator may exert its effects directly on the trabecular cells or that it may act indirectly through upregulating the production of TGF-β1. Invest Ophthalmol Vis Sci. 1996;37:2778–2782.

Transforming growth factor-β (TGF-β) is a family of multifunctional regulatory proteins. Of the five closely related homologous TGF-β isoforms, only TGF-β1, -β2, and -β3 are expressed in mammalian tissue.1 All three isoforms have been detected in ocular tissues of the anterior segment, and both TGF-β1 and -β2 are found in normal aqueous humor of the human eye.2–4 Earlier studies also revealed that trabecular cells express high-affinity receptors (types II and III) for TGF-β, which inhibits the mitotic and the migratory activities of these neural crest-derived cells and enhances their production and secretion of fibronectin in vitro.5–7

In several normal and transformed cell lines, such as lung fibroblasts, rat kidney fibroblasts, fibrosarcoma, and osteoblast-like cells, TGF-β1 positively regulates its own gene expression and increases the secretion of TGF-β1 protein in the culture medium of the treated cells.6 TGF-β1 and -β2 upregulate the expression of all three isoforms in a mouse fibroblastic cell line.9 If this phenomenon holds true for trabecular cells, the increased concentration of TGF-β2 in the aqueous humor of patients with glaucoma could be sustained, and the inhibitory effect of TGF-β on trabecular cell growth, as well as its enhancing effect on the production of extracellular matrix components, amplified,8 could contribute to the pathophysiologic changes of the aqueous outflow pathway in patients with glaucoma.

During the past few years, polymerase chain reaction (PCR) techniques that use some form of internal standard have been developed to quantitate messenger RNA levels.10 In multiplex PCR, the internal standard is a ubiquitously expressed housekeeping gene, such as glyceraldehyde-3-phosphate dehydrogenase (G3PDH), that is coamplified with the target gene. The authors previously showed that the level of TGF-β2 in the aqueous humor of glaucomatous eyes is significantly higher than that of age-matched nonglaucomatous controls. The current finding suggests that this growth modulator may exert its effects directly on the trabecular cells or that it may act indirectly through upregulating the production of TGF-β1. Invest Ophthalmol Vis Sci. 1996;37:2778–2782.

A PCR mimic for TGF-β2 is not commercially available at present, though techniques have been reported to construct competitive templates. The mimic for TGF-β1 is obtainable from commercial suppliers, and we investigated the effect of TGF-β1 and -β2 on the messenger RNA expression of TGF-β1 in the first-passage cultures of porcine trabecular cells by semiquantitative PCR.

MATERIALS AND METHODS. Cell Culture. Eagle’s minimal essential medium and newborn calf serum were purchased from Gibco BRL Life Technologies (Gaithersburg, MD). Recombinant human basic fibroblast growth factor (bFGF), ultrapure natural human TGF-β1, and recombinant human TGF-β2 were obtained from Genzyme Diagnostics (Cambridge, MA). Cultures of trabecular cells were initiated from fresh porcine eyes procured within 20 minutes of decapitation, as described previously,13–14 and the investigation adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. After 3 to 4 weeks’ incubation in tissue culture medium (Eagle’s minimal essential medium supplemented with 15% newborn calf serum), when the cultures were near confluent, the explants were removed, and the trabecular cells were trypsinized. Trabecular cells were subcultured in tissue culture medium for 24 hours, rendered quiescent by incubation in serum-free culture medium (SFM) for 48 hours, and treated for 1 hour with bFGF (1 ng/ml) in SFM, TGF-β1 (1 ng/ml, active form) with bFGF (1 ng/ml, active form) in SFM, TGF-β2 (1 ng/ml, active form) in SFM, 5% newborn calf serum in Eagle’s minimal essential medium, or fresh SFM as controls. For each condition, triplicate experiments were performed.

Complementary DNA Preparation. The extraction of total cellular RNA and synthesis of first-strand cDNA were performed as described previously,15 except that an oligo(dT)15 primer instead of specific downstream primers was used for the cDNA synthesis in the current experiment. The amount of RNA was determined by using a Spectronic 21 UVD spectrophotometer (Milton Roy, Rochester, NY) at a wavelength of 260 nm, and all cDNA synthesis reactions were performed with 1 µg total RNA.

Semiquantitative Polymerase Chain Reaction. Multiplex PCR and competitive PCR were performed on aliquots of cDNA by using either an endogenous (G3PDH) or exogenous sequence (PCR mimic for TGF-β1) as internal standards, respectively.

The PCR primers and TGF-β1 mimic were purchased
from Clontech Laboratories (Palo Alto, CA). The sequences of the primers specific for the target genes were:

**TGF-β1** (expected size of product, 161 bp for products obtained from cDNA sample and 300 bp for those obtained from the TGF-β1 mimic),

5'-GCCCTGGACACCAACTATTGCT-3' (upstream primer),
5'-AGGCTCCAAATGTAGGGGCAGG-3' (downstream primer);

**G3PDH** (expected size of product, 450 bp),
5'-ACCACAGTCCATGCCATCAC-3' (upstream primer),
5'-TCCACACCCCTGTTGCTGTA-3' (downstream primer).

For multiplex PCR, the same amount of cDNA (one tenth of the cDNA reaction products) was used

in each sample, and for competitive PCR, the same amount of TGF-β1 mimic (3 × 10⁻³ attomole) was spiked in each reaction tube. The PCR reaction was performed for 30 cycles based on results from preliminary experiments.

**Laser Densitometry.** We performed quantitative analysis of the PCR products by densitometric scanning of the photographic negatives obtained from the ethidium bromide-stained agarose gels with a HeNe laser densitometer (Pharmacia LKB Biotechnology,
Piscataway, NJ) and calculated the ratios of the product of interest to that of either G3PDH or the TGF-β1 mimic. Student's t-test was used to determine the statistic significance of the findings.

RESULTS. For multiplex PCR using both primer pairs of TGF-β1 and G3PDH, two products were obtained for each sample studied (Fig. 1). The size of the products (161 bp and 450 bp), as determined by regression analysis with the standard molecular size markers (Phi X 174 DNA/Hae III size markers), corresponded to that of the expected product of TGF-β1 and G3PDH, respectively. The negative control reaction, without the addition of templates, did not reveal any amplification. Densitometric analysis showed that TGF-β1 (1 ng/ml) treatment for 1 hour increased its own gene expression 2.97-fold over the untreated control cells (Fig. 2), and this effect was statistically significant (P < 0.01).

For competitive PCR experiments, two amplified products with the anticipated length of 300 bp and 161 bp (corresponding to the products of TGF-β1 mimic and TGF-β1 mRNA, respectively) were obtained (Fig. 3). In the negative control, no amplified fragments were observed. Compared with cells incubated in SFM, exposure to TGF-β1 or β2 increased TGF-β1 messenger RNA level 3.07- and 2.82-fold, respectively (P < 0.01). No significant effect was observed in trabecular cells incubated in 5% newborn calf serum or treated with bFGF (P > 0.05) (Fig. 4).

DISCUSSION. Both multiplex PCR and competitive PCR amplified two products with the expected size based on the location of the primer pairs, indicating that these sequences are specific for TGF-β1 and G3PDH or TGF-β1 mimic. The two techniques yielded similar results for the actions of TGF-β1 on its own messenger RNA expression, which leads us to believe that our results are valid and that both techniques, when used appropriately, can be used reliably in experiments requiring relative semiquantitative studies.

TGF-β1 positively regulates its own mRNA expression in cultured trabecular cells of the first passage. Considering the multifunctional actions of this cytokine, which include increased production and deposition of extracellular matrix material and growth inhibition of trabecular cells, a change in the concentration of the activated form of TGF-β within the eye would have profound effects because of this unique autoinductive activity.

Transforming growth factor-β2 treatment also increased TGF-β1 gene expression. We showed previously that the levels of total and intrinsically active forms of TGF-β2 in the aqueous humor of glaucomatous eyes are significantly higher than those of age-matched, nonglaucomatous controls. These findings have been confirmed by several other groups of investigators. Our current results suggest that this growth modulator may exert its effects directly on the trabecular cells and indirectly by upregulating the production of TGF-β1. Because TGF-β enhances the synthesis and secretion of several extracellular matrix components, such as fibronectin, and inhibits trabecular cell proliferation and migration in vitro, this cytokine is implicated in the pathogenetic process of primary open-angle glaucoma, a disease characterized by the abnormal buildup of extracellular matrix components and decreased cellularity in the trabecular meshwork–Schlemm’s canal system. Future studies will be conducted on developing new therapeutic modalities for glaucoma by focusing on TGF-β antagonism. This is a promising approach because neutralizing antibodies against TGF-β will inhibit effectively the autocatalytic and autoinductive cascades of TGF-β amplification, decreasing the eventual growth factor concentration and the extent of its undesirable actions.

Key Words
cell culture, gene expression, growth factor, laser densitometry, quantitative polymerase chain reaction

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References
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FIGURE 4. Ratios of polymerase chain reaction products for transforming growth factor (TGF)-β1 over those for TGF-β1 mimic from cells incubated in serum-free medium or treated with 5% newborn calf serum, basic fibroblast growth factor, TGF-β1, and TGF-β2 after densitometric quantitation. Error bars represent the standard error of the mean from three independent experiments.


