Effects of TGF-β2 on Immune Response–Related Gene Expression Profiles in the Human Corneal Endothelium

Satoru Yamagami,1 Seichi Yokoo,1 Tatsuya Mimura,2 and Shiro Amano2

PURPOSE. To determine the effects of transforming growth factor (TGF)-β2 on immune-response–related gene expression profiles in the stimulated human corneal endothelium (HCE).

METHODS. A human complementary DNA (cDNA) expression array analysis was used to investigate the effects of TGF-β2 on cultured HCE incubated with interleukin (IL)-1α and tumor necrosis factor (TNF)-α. Gene-specific semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) were performed to examine the gene expression patterns revealed by the cDNA expression array analysis. Moreover, the expression of newly identified genes in HCE was confirmed by RT-PCR in human donor corneas.

RESULTS. cDNA expression array analysis and semiquantitative RT-PCR revealed that TGF-β2 downregulated the expression of IL-6, growth-related (Gro)-α (CXCL1), monocyte chemotactic protein (MCP)-1 (CCL2), granulocyte-colony stimulating factor (G-CSF), and insulin-like growth factor binding protein (IGFBP)-5 and upregulated the expression of tissue inhibitor of metalloproteinase (TIMP)-1. ELISA confirmed TGF-β2-mediated changes in the expression of IL-6, CXCL1, CCL2, G-CSF, IGFBP-5, and TIMP-1 at the protein level. CXCL1, G-CSF, and IGFBP-5 mRNAs were detected for the first time in the HCE of donor corneas.

CONCLUSIONS. TGF-β2 downregulates IL-6, CXCL1, CCL2, G-CSF, and IGFBP-5, and upregulates TIMP-1 in cultured HCE stimulated with proinflammatory cytokines, suggesting the immunomodulatory role of TGF-β2 in the aqueous humor and the pathophysiological significance of TGF-β2 in the anterior chamber of the eye. (Invest Ophtalmol Vis Sci. 2004;45: 515–521) DOI:10.1167/iovs.03-0912

The corneal endothelium (CE) faces the aqueous humor (AH) as a part of the inner side of the anterior chamber of the eye. The CE constitutively expresses various genes to maintain fundamental corneal functions, such as stromal dehydration, corneal transparency, metabolic activity, and signal transduction.1,2 Moreover, ex vivo and cultured human CE (HCE) cells express abundant cytokine and chemokine genes by cytokine stimulation.3 Because it has long been recognized that the anterior chamber of the eye has an immune privilege, analysis of the interaction between CE and AH can provide a theoretical rationale for the immune-privileged status in the anterior segment of the eye.

Transforming growth factor (TGF)-β is a stable, multifunctional polypeptide growth factor.4 Specific receptors for this protein have been found on various cells, but the effect of the molecule varies with the cell type and growth conditions. There are three isoforms of TGF-β. They have similar, but not identical, functions on target cells.4 In the AH of the eye, various immunosuppressive substances, such as TGF-β, α-melanocyte stimulating hormone,5 vasoactive intestinal peptide,6 and macrophage migration inhibitory factor,7 have the capacity to modulate immune responses.8 Among them, abundant TGF-β2, which is produced in the ciliary epithelium,9 is secreted into the normal AH10,11 and is considered to play a critical role in the immune privilege of the anterior chamber.11 It is not fully understood, however, how TGF-β2 affects immunoreactive factors produced in the AH.

In this study, we prepared HCE cells stimulated with interleukin (IL)-1α and tumor necrosis factor (TNF)-α, because the cornea synthesizes these proinflammatory cytokines in inflamed conditions such as uveitis,12,13 herpetic stromal keratitis,14 and corneal allograft rejection.15,16 We then investigated the effects of the active form of TGF-β2 on the immune-response–related gene expression profiles in the HCE with a complementary DNA (cDNA) expression array analysis. Our findings revealed that TGF-β2 regulates immune-response–related genes in the HCE that are associated with the correlation between the HCE and AH in the anterior chamber of the eye.

MATERIALS AND METHODS

Cell Culture and Cytokine Treatment

Normal human donor corneas were supplied by the Central Florida Lions Eye and Tissue Bank. The HCE and Descemet membrane were peeled away in a sheet from the periphery to the center of the cornea with fine forceps. To avoid the inclusion of posterior stromal tissue, only strips of HCE-Descemet tissue that were excised smoothly from the stroma, without residual stroma, were used in the experiments. Primary HCE cells were cultured as described elsewhere.17 Recombinant human IL-1α, TNF-α, and TGF-β2 (R&D Systems, Minneapolis, MN) were diluted with phosphate-buffered saline (PBS). Fifth-passage HCE cells were transferred to 2 mL of DMEM containing 1% fetal bovine serum in 50-mm culture dishes and then treated for 24 hours with 20 ng/mL each of human IL-1α and TNF-α (IL-1α/TNF-α group), 20 ng/mL each of human IL-1α and TNF-α plus 20 ng/mL of recombinant human TGF-β2 (TGF-β2 group), or PBS only (control group). Two independent culture dish sets were prepared. RNAs isolated from those groups were used for gene array analysis or semiquantitative RT-PCR. The supernatants were stored at −70°C until use in an enzyme-linked immunosorbent assay (ELISA).

Human Immunology Gene Array

A human cDNA expression array (Toyobo, Osaka, Japan), in which 648 known immunology-related genes are represented, was used in this experiment. A complete list of the 648 immunology-related genes
included in this human array can be found at http://www.toyobo.co.jp/seihin/hr/index.html. Total RNA was isolated using an RNA extraction reagent (Isogen; Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. After DNase treatment, an α-<sup>32</sup>P-labeled cDNA probe was synthesized from the total RNA. Each cDNA probe was purified (Chroma-spin columns; Clontech, Palo Alto, CA). Incorporation of the label was assessed by scintillation counting. Equal counts per minute of the cDNA probe for each group was hybridized in a hybridization solution (Express Hyb; Clontech) with the human gene immunology array membranes overnight at 68°C with continuous agitation. The array membranes were washed in wash solution 1 (2× SSC, 1% SDS) and then in wash solution 2 (0.1× SSC, 0.5% SDS) at 68°C. The array membranes were exposed to a phosphorescence imager (Eastman Kodak, Rochester, NY) at room temperature. Two sets of phosphorescent imaging results on separate culture experiments were analyzed (four hybridizations of four cDNA array membranes) and compared with those from another phosphorescence imager (Storm PhosphorImager; Amersham Biosciences, Sunnyvale, CA). The data obtained from the phosphorescence imaging analysis system reflects the accumulated x-ray energy and provides us with accurate quantification in comparison with densitometric analysis with spot size. After normalization to the intensity levels of all housekeeping genes included in the membrane, the average intensity differences among genes upregulated or downregulated in the two hybridizations were calculated (Vision Array, ver. 1.1 software; Imaging Research Inc., Ontario, Canada).

RNA Preparation and RT-PCR

Total RNA was isolated from cultured HCE cells and from HCE peeled from donor corneas, by use of an extraction reagent (Isogen; Nippon Gene) according to the manufacturer’s instructions. Water was used as a negative control. After DNase treatment, first-strand cDNA was synthesized from the total RNA with a reverse-transcription system (Promega Corp., Tokyo, Japan). The PCR reaction mixtures comprised 1% cDNA, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 20 pmol oligonucleotides, and 2.5 U Taq polymerase (AmpliTaq Gold; Perkin Elmer, Wellesley, MA), in a 50-μL reaction volume. After incubation at 95°C for 9 minutes, amplification was performed at 94°C for 30 seconds and then at 60°C for 30 seconds in a thermocycler (T Gradient Bio-Rad Laboratories, Hercules, CA). Samples were separated in a 2% agarose gel, and the products were visualized with ethidium bromide. An optical scanner was used to determine the densities of the gel bands of the PCR products. The band densities were normalized to those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The concentration of tissue inhibitor of matrix metalloproteinase (TIMP)-1 was quantitated with an ELISA kit (Duoset ELISA Development Kit; R&D Systems) and plates read with a microplate reader (Molecular Bioscience Group, Hercules, CA) at an optical density of 450 nm, with the correction wavelength set at 550 nm.

Detection of CXCL1, G-CSF, and IGFBP-5 Expression in Ex Vivo HCE Cells from Donor Corneas

To confirm the expression of newly identified genes CXCL1, G-CSF, and IGFBP-5 expression in HCE cells, RT-PCR was performed in HCE of donor corneas immersed in storage medium (Optisol GS; Chiron Vision, Irvine, CA). The HCE was peeled off as a sheet and subjected to mRNA extraction, DNase treatment, and subsequent RT-PCR. Water was used as a negative control for RT-PCR.

Statistical Analysis

The Fisher protected least-significant-difference test was used to compare the band densities on RT-PCR, and the protein concentrations on ELISA. P < 0.05 was considered significant.

ELISA of Supernatants of Cultured HCE with or without TGF-β2 Stimulation

Culture supernatants were used for ELISA. The protein concentrations of IL-6, monocyte chemotactic protein 1 (MCP-1, CCL2), granulocyte-colony stimulating factor (G-CSF; Biosource International, Camarillo, CA), and Gro-α (CCL1; R&D Systems) in the culture supernatant were measured with ELISA kits, according to the manufacturer’s protocol. The insulin-like growth factor binding protein (IGFBP)-5 concentration was measured by direct ELISA. Recombinant IGFBP-5 (R&D Systems) and diluted serum were incubated at room temperature for 2 hours in a 96-well coated plate. Polyclonal goat anti-human IGFBP-5 antibody (1 μg/mL; R&D Systems) was applied for 1 hour as a detection antibody after blocking was performed with 3% bovine serum albumin at room temperature for 30 minutes. A horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (H+L) antibody (1 μg/mL; Bethyl Laborato-

![Figure 1](https://example.com/figure1.jpg)  
**Figure 1.** Representative human immunology-related gene arrays hybridized with α-<sup>32</sup>P-labeled cDNA probes synthesized from total RNA isolated from cultured HCE exposed to (A) 20 ng/mL each of IL-1α and TNF-α (IL-1α/TNF-α group) and to (B) 20 ng/mL each of IL-1α, TNF-α, and TGF-β2 (TGF-β2 group) for 24 hours. The TGF-β2-downregulated genes were IL-6, Groα (CXCL1), MCP-1 (CCL2), IL-8 (CXCL8), G-CSF, IGFBP-5, and C/EBP-β. The TGF-β2-upregulated gene was TIMP-1. 1: IL-6, 2: CXCL1, 3: CCL2, 4: CXCL8, 5: G-CSF, 6: IGFBP-5, 7: C/EBP-β, 8: TIMP-1. Data are representative of two independent experiments.
RESULTS

Screening of Immune-Response–Related Gene Expression by cDNA Expression Array

A series of experiments was performed twice using RNA isolated from two separate cultures of either IL-1α/TNF-α or TGF-β2 group (four hybridizations of four cDNA array membranes). The far left and far right duplicate lanes of these membranes contained 11 housekeeping gene controls and two negative controls. No signals were observed for the negative membranes. The far left and far right duplicate lanes of these membranes (Fig. 1; Table 1). The average intensity differences greater than 1.5 (in arbitrary units) between the IL-1α/TNF-α–treated and IL-1α/TNF-α plus TGF-β2–treated groups were accepted as significant and applied for further study.

TABLE 1. Genes Downregulated or Upregulated on TGF-β2 Treatment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Intensity Difference</th>
<th>GenBank Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Downregulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin 6 (IL-6)</td>
<td>−53.0</td>
<td>M29150</td>
</tr>
<tr>
<td>CXCL1, growth-related α (GROα)</td>
<td>−38.4</td>
<td>NM001511</td>
</tr>
<tr>
<td>CCL2, monocyte chemotactic protein (MCP-1)</td>
<td>−19.6</td>
<td>X14768</td>
</tr>
<tr>
<td>CXCL8, IL-8</td>
<td>−15.0</td>
<td>X00787</td>
</tr>
<tr>
<td>Granulocyte-colony stimulating factor (G-CSF)</td>
<td>−12.1</td>
<td>X03655</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein (IGFBP)-5</td>
<td>−8.0</td>
<td>NM00599</td>
</tr>
<tr>
<td>CCAAT/enhancer binding protein (C/EBP) β</td>
<td>−7.1</td>
<td>X52560</td>
</tr>
<tr>
<td>Siva</td>
<td>−2.3</td>
<td>U82938</td>
</tr>
<tr>
<td>Prolactin</td>
<td>−2.2</td>
<td>V00566</td>
</tr>
<tr>
<td>Indian hedgehog protein (Ihh)</td>
<td>−2.0</td>
<td>L38517</td>
</tr>
<tr>
<td>Fibroblast growth factor (FGF)-7</td>
<td>−1.8</td>
<td>M60828</td>
</tr>
<tr>
<td>IGFBP-4</td>
<td>−1.8</td>
<td>M62403</td>
</tr>
<tr>
<td>MMP2 (type IV collagenase, gelatinase A)</td>
<td>−1.7</td>
<td>J03210</td>
</tr>
<tr>
<td>Insulin receptor substrate (IRS)-2</td>
<td>−1.6</td>
<td>AF073510</td>
</tr>
<tr>
<td>Glucose-regulated protein (GRP)</td>
<td>−1.5</td>
<td>X15187</td>
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<tr>
<td>Upregulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue inhibitor of matrix metalloproteinase (TIMP)-1</td>
<td>10.6</td>
<td>X03124</td>
</tr>
<tr>
<td>Extracellular signal-regulated kinase (ERK) 5</td>
<td>3.2</td>
<td>U25278</td>
</tr>
</tbody>
</table>

The two sets of autoradiograph results were analyzed and compared. The average intensity differences greater than 1.5 (in arbitrary units) between the IL-1α/TNF-α–treated and IL-1α/TNF-α plus TGF-β2–treated groups are listed.

Immune-Response–Related Gene Expression by Semiquantitative RT-PCR Analysis

To confirm the results of gene array analysis and examine whether TGF-β2 antagonizes or enhances effects of IL-1α and TNF-α, semiquantitative RT-PCR was performed. The oligonucleotide primers used for RT-PCR are listed in Table 2. GAPDH was detected on RT-PCR in the control, IL-1α/TNF-α, and TGF-β2 groups. There were no significant differences in GAPDH among the groups (Fig. 2A). After confirmation of linear amplification of the PCR products, as shown in Figure 2A, the gene expression levels were compared. Figure 2B

TABLE 2. Oligonucleotide Primers for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR Primers</th>
<th>Product Size (bp)</th>
<th>GenBank Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-GGTGAGGGTGCTGTAAGCCGA-3'</td>
<td>225</td>
<td>P04406</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-GTTAGGCTGGGCTACCTCCTTC-3'</td>
<td>522</td>
<td>Y00562</td>
</tr>
<tr>
<td>CXCL1 (GROα)</td>
<td>5'-TTACAGACACAGAATGCCC-3'</td>
<td>530</td>
<td>NM001511</td>
</tr>
<tr>
<td>CCL2 (MCP-1)</td>
<td>5'-CCCCTGACCAAAACATGCTTA-3'</td>
<td>267</td>
<td>M24545</td>
</tr>
<tr>
<td>CXCL8 (IL-8)</td>
<td>5'-AAGGCCGGAAGGCAACCT-3'</td>
<td>466</td>
<td>Y00787</td>
</tr>
<tr>
<td>G-CSF</td>
<td>5'-GGCCAGGACATGGAAGAATC-3'</td>
<td>263</td>
<td>X03655</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>5'-CCCTGAGGCTGCTGGCATGAA-3'</td>
<td>371</td>
<td>NM00599</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>5'-GAAGCCCGGCTGTAATTTT-3'</td>
<td>309</td>
<td>X52560</td>
</tr>
</tbody>
</table>

5' - 3' orientation of the primers relative to the 5' and 3' ends of the genes.
Altered protein production. Figure 3 shows that six proteins in the IL-1α/TNF-α group were significantly higher than those in the control group. The levels of CXCL1, CCL2, G-CSF, and IGFBP-5 production in the TGF-β2 group were significantly lower than those in the IL-1α/TNF-α group. On the contrary, the level of TIMP-1 in the TGF-β2 group was significantly higher than that in the IL-1α/TNF-α group.

CXCL1, G-CSF, and IGFBP-5 Expression in Ex Vivo HCE by RT-PCR Analysis

RT-PCR was performed to detect mRNA expression of the newly identified CXCL1, G-CSF, and IGFBP-5 gene expression in ex vivo HCE cells from donor corneas. Two donor corneas were examined separately. GAPDH was detected in ex vivo HCE of donor corneas, but not in a negative control sample. CXCL1, G-CSF, and IGFBP-5 mRNAs were all detected in ex vivo HCE cells of two donor corneas (Fig. 4).

Discussion

In the present study, we conducted a comprehensive examination of the effects of TGF-β2 on the expression of immune-response–related genes in HCE cells stimulated with the proinflammatory cytokines IL-1α and TNF-α, using a combination of cDNA array and semiquantitative RT-PCR analysis. We demonstrated that TGF-β2 downregulated the expression of IL-6, Groα (CXCL1), CCL2, G-CSF, and IGFBP-5, and upregulated the expression of TIMP-1. The number of TGF-β2–downregulated genes was higher than those upregulated by TGF-β2, consistent with the effect of TGF-β1 on the gene expression profiles in cultured human corneal epithelium. Among the TGF-β2–downregulated genes, detected by cDNA array and RT-PCR analysis, the expression levels of IL-6, CXCL1, CCL2, G-CSF, and IGFBP-5 were also confirmed by ELISA analysis. The TIMP-1 gene and protein were upregulated with TGF-β2 treatment. Moreover, in addition to the expression of IL-6, CCL2, CXCL8, and TIMP-1 in HCE, as previously described, our data showed for the first time that HCE expresses CXCL1, G-CSF, and IGFBP-5 mRNAs. Although the donor corneas were

Immune-Response–Related Protein Production

We next determined whether the change in IL-6, CXCL1, CCL2, G-CSF, IGFBP-5, and TIMP-1 mRNA expression results in altered protein production. Figure 3 shows that six proteins in the IL-1α/TNF-α group were significantly higher than those in the control group, consistent with the results of semiquantitative RT-PCR. The high amount of IL-6 produced in the IL-1α/TNF-α group was significantly suppressed in the TGF-β2 group. The levels of CXCL1, CCL2, G-CSF, and IGFBP-5 production in the TGF-β2 group were significantly lower than those in the IL-1α/TNF-α group. On the contrary, the level of TIMP-1 in the TGF-β2 group was significantly higher than that in the IL-1α/TNF-α group.
the existence of unidentifiable blood mononuclear cells. In addition, CD4 T cells exposed to CD4 T cells have been observed in human G-CSF and CD4 T cells require an immunosuppressive effect on allogeneic immune responses. TGF-β has no effect on ovine articular chondrocyte IGFBP-5. In the anterior segment of the eye, IGFBP-5 has been detected in the rat ciliary process and the human trabecular meshwork. In our study, IGFBP-5 was detected in cultured and ex vivo HCE cells. The exact role of IGFBP-5 in HCE is, however, still unknown in the anterior chamber of the eye. Further study is needed to reveal the function of IGFBP-5 in the AH.

IL-6 is a pleiotropic cytokine whose synthesis can be induced by IL-1α and TNF-α. IL-6 was detected in the AH of patients and rodents with uveitis, and thus it has been considered a major mediator of uveitis. IL-6 is not by itself mitogenic for T-cell proliferation, but can antagonize the immunosuppressive effect of the active form of TGF-β2, which potently suppresses T-cell proliferation. The suppression of IL-6 secretion with the active form of TGF-β2 in HCE implies that TGF-β2 can indirectly regulate IL-6 in the AH, although secreted IL-6 by itself antagonizes the immunosuppressive effect of TGF-β2.

Matrix metalloproteinase (MMP) activity is controlled through interaction with TIMPs, principal natural inhibitors of MMPs. PMNs lead to tissue damage through the activation and secretion of MMPs, while promoting bacterial clearance. TIMP-1 prevents infection by protecting against tissue destruction, and TGF-β1 and -β2 have been reported to induce expression of the TIMP-1 gene. Upregulation of TIMP-1 in the HCE by TGF-β2 in the AH may indicate a mechanism that minimizes the destructive damage in the HCE.

It is worth commenting on the modulation of angiogenesis by cytokines, a central process in physiological and pathophysiological situations. Angiogenesis, a common feature of inflammatory, infectious, and traumatic diseases of the cornea, leads to visual impairment and failure of the immune privilege of the cornea. In the anterior chamber of the eye, TGF-β2 is regarded as a critical factor that protects the corneal tissue against abnormal vascularization. G-CSF and CXCL1 regulate the expression of TIMP-1, which are expressed abundantly in the HCE, exhibit potent angiogenic activity. On the contrary, TIMP-1 suppresses angiogenesis in the cornea. Our findings that TGF-β2 downregulates G-CSF and CXCL1, and upregulates TIMP-1 expression may explain the antiangiogenic effect of TGF-β2 in the AH.

In summary, we investigated the effect of TGF-β2 on the immune-response-related genes expressed in the HCE, using cDNA array technology, semiquantitative RT-PCR, and ELISA. TGF-β2 downregulates IL-6, CXCL1, CCL2, G-CSF, and IGFBP-5, and upregulates TIMP-1 in cultured HCE stimulated with proinflammatory cytokines, indicative of immunomodulatory roles of TGF-β2 in the AH. Furthermore, we identified, for the first time, CXCL1, G-CSF, and IGFBP-5 expressed in cultured and ex vivo HCE cells. Our results have pathophysiological implications in the anterior chamber immunology of the eye.

Acknowledgments

The authors thank Chintatsu Kamikokuryo-Yokota for excellent technical support, Koichi Ohta and Nobuyuki Ebihara for helpful suggestions on TGF-β stimulation experiments, and IOVS volunteer editor Dongli Yang (University of Michigan, Ann Arbor, MI) for editing the manuscript.
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