The Effect of TGF-β1 on Differential Gene Expression Profiles in Human Corneal Epithelium Studied by cDNA Expression Array

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Purpose. TGF-βs regulate cell proliferation and differentiation, and they play important roles in maintenance of corneal epithelium. However, the precise function of TGF-βs in the corneal epithelium remains unclear. In this study, cDNA expression array technology was used to demonstrate the effect of TGF-β1 on the simultaneous expression of a large number of genes in cultured human corneal epithelial cells (HCECs). The change in protein level expression of the specific genes influenced by TGF-β1 was also investigated.

Methods. Human cDNA expression array technology was used to study the simultaneous expression of 1176 specific cellular genes in HCECs incubated with TGF-β1 (10 ng/ml). Moreover, gene-specific semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) was used to confirm the gene expression pattern measured by the cDNA expression array. Western blot analysis was used to examine protein expression of the specific genes in the presence or absence of TGF-β1.

Results. TGF-β1 significantly upregulated the expression of 19 genes and significantly downregulated ras-related protein, caspase10, and β4-integrin in the treated HCECs. The expression of 277 genes including α3-integrin, PAI-2, transferrin receptor, and cyclin-D1 was studied. Semiquantitative RT-PCR analysis confirmed the TGF-β1–mediated changes in expression patterns of these genes. Furthermore, Western blot analysis revealed that TGF-β1 remarkably decreased PAI-2, transferrin receptor, and integrin α3, and increased caspase10 on the protein level.

Conclusions. TGF-β1 regulates the expression of specific types of genes in HCECs. These results strongly suggest that TGF-β1 is critically involved in the maintenance of the corneal epithelium through the control of a network of various signal-transduction pathways. (*Invest Ophthalmol Vis Sci.* 2001;42: 1691–1697)

The cornea is maintained by various factors and mechanisms associated with the regulation of its epithelial cells. For example, in response to corneal injury, epithelial cells migrate from nonwounded sites to cover the denuded area and then proliferate to form a stratified layer. Corneal regeneration of the damaged area with a new epithelium requires the migration, proliferation, and differentiation of corneal epithelial cells. Moreover, the inhibition of proliferation and differentiation of epithelial cells at some stages is required to prevent the cornea from forming an excess amount of scar.[1–3] This negative reaction is also important in recovering appropriate morphology and function of the cornea. Such processes depend on the organized activities of a variety of cytokines, such as growth factors interleukins, and of extracellular matrix proteins.

One of the most important mediators in the wound-healing process is the family of transforming growth factor (TGF)-βs.[4–7] TGF-βs, a family of cytokines, have multifunctional regulatory activities. As mediators that draw fibroblasts and macrophages into an inflammatory focus, they regulate cell growth and differentiation, control the immune system, and stimulate extracellular matrix production.[8,9] Previous reports have suggested that TGF-β inhibits proliferation of corneal epithelial cells either in vivo or in vitro, but has no effect on their migration or adhesion.[10–12] In addition, it has been demonstrated that TGF-β antagonizes the actions of epidermal growth factor (EGF) on corneal epithelial cells that stimulate corneal epithelial proliferation.[12,13] However, the coordinated changes of mRNA levels in corneal epithelium under different conditions remain unknown.

The TGF-β family is composed of five isoforms (TGF-β1–5).[8,14,15] Of these isoforms, TGF-β1, -β2, and -β3 are found in mammals, including humans.[8] These isoforms are present in the corneal epithelium at the mRNA and/or protein levels.[9,10,16,17] In normal corneal epithelium, TGF-β receptors type I and II act as transmembrane serine-threonine kinases and are responsible for signal transduction. Both receptor types are present in basal cells of the corneal epithelium. Type III receptor, a proteoglycan that may regulate the ligand-binding ability or surface expression of the type II receptor,[18,19] was detected in all cell layers of the corneal epithelium. Several reports have indicated the effect of TGF-βs in corneal epithelial wound healing.[10,13,20] However, the specific roles of the three specific TGF-βs have not been clarified. Moreover, the functional differences among TGF-β isoforms in corneal epithelial wound healing remain unknown.

Recently, powerful tools have been developed for parallel analysis of mRNA expression of a large number of genes.[21–23] cDNA arrays offer the potential to quantify simultaneous expression of many genes. cDNA arrays have the obvious advantage of allowing the analysis of multiple clones and large-scale comparison of multiple nucleic acid sequences with a single hybridization. Furthermore, progress in addressing issues such as probe density, probe content, array size, and data analysis has rendered this technology sufficiently flexible and accessible for application in the laboratory.[24] Increases in sensitivity have enhanced the detection resolution to the level of a single mRNA copy per cell for genome-wide transcriptional analysis. With this technology, the previously unknown regulatory functions of various molecules, such as TGF-β, can be detected. We

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report the effect of TGF-β1 treatment on gene expression in cultured human corneal epithelial cells (HCECs). We show that TGF-β1 significantly affects the expression levels of nearly 300 genes. Remarkably, the majority of them are downregulated.

Materials and Methods

Cell Culture

Normal HCECs, which had been previously frozen after primary culture, were obtained from Kurabo (Osaka, Japan). The cells were thawed and cultured in 25-cm^2 culture flasks (Corning Laboratories, Corning, NY), in Medium 165 (serum-free medium, specific for HCECs, containing 5 μg/ml insulin, 0.18 μg/ml hydrocortisone, 5 μg/ml transferrin, 12.5 μg/ml amphotericin B, 0.15 mM Ca^{2+}, 5000 U/ml penicillin G, 1 ng/ml EGF, and 0.4% bovine pituitary extract; Kurabo). The cells were fed every 2 days with the culture medium and subcultured into 75-cm^2 culture flasks. Cells from the fourth passage were used in the experiments. Each flask contained 8 ml culture medium without any growth factors, serum, or other extracts. The cells were incubated at 37°C with 5% CO_2. Human recombinant TGF-β1 (Roche Diagnostics, Mannheim, Germany) was added to the medium (final concentration, 10 ng/ml). Because the effect of TGF-β1 plateaus after 12 hours, the cells were washed twice in PBS and then harvested after 12 hours.

RNA Isolation

Total RNA was isolated with a kit (Isogen kit; Nippon Gene, Tokyo Japan), according to the manufacturer's instructions. Purification of total RNA using DNase I treatment was performed (Atlas Pure Total RNA Isolation kit; Clontech, Palo Alto, CA) according to the manufacturer's instructions. Purified total RNA (20 μg) was used for $polyA^+$ RNA enrichment with the same kit. RNA concentrations were calculated from absorbance at 260 nm.

cDNA Synthesis and Hybridization

Human cDNA expression array (Atlas Human 1.2 Array: Clontech) was used to compare differential gene expression between TGF-β1–treated and untreated HCEC cultures. The array membrane contained the cDNAs of 1176 known genes and 9 housekeeping genes. The complex ^{32}P-labeled first-strand cDNA probes were synthesized from $polyA^+$ RNA obtained from normal and TGF-β–treated cells by reverse transcription in the presence of (α-^{32}P) dATP, and they were purified according to the protocol provided in the user manual. Briefly, after the denaturation step, cDNAs were synthesized by incubation at 50°C for 25 minutes in a master mix (total reaction volume; 11.5 μl) containing 2 μl dNTP (500 μM, without dATP), 5 μl (α-^{32}P) dATP (3000 Ci/mmol; Amersham, Cleveland, OH) and 1600 units of Maloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in 1× reverse transcription buffer. The reaction was terminated by heating for 5 minutes at 70°C, and unincorporated nucleotides were removed by spin-column purification. For each reaction, 2 to 10 × 10^6 counts per minute (cpm) was incorporated into the final product. After purification, labeled cDNAs were denatured by boiling for 5 minutes and then hybridized onto the human cDNA array blots in a hybridization solution (~2 × 10^6 cpm/ml; ExpressHyb hybridization solution, Clontech). The membranes were prehybridized with the hybridization solution without the probe at 68°C for at least 2 hours before the probe was added.

The hybridization was performed at 68°C in a roller bottle overnight. After two washes with 2× SSC and 0.1% SDS at 68°C for 20 minutes, the membranes were subjected to a stringent wash with 0.1× SSC, 0.5% SDS, and 0.1 mM EDTA at 68°C. After hybridization and washing, the array filters were sealed in plastic bags and exposed to a phosphorimaging screen for 24 hours at room temperature. The exposed screens were scanned with an image analysis system (Phosphorimager Fuji MCID-BAS; Fuji Bio-Imaging Analyzer BAS2000; Fuji Film, Tokyo, Japan), and the spots on the array images were quantified on computer (ArrayGauge software; Fuji Film). The grid was superimposed over the array image, with each box in the grid containing a single array element. The median count within each box was recorded and corrected by subtracting its local background. The signal intensity of each single spot was scanned and normalized to the expression of all nine housekeeping genes. Changes in the expression levels of the various genes were then calculated by densitometric scanning of the hybridized signals and provided in photograph-stimulated luminescence (PSL) units using a software program (Array Gauge software; Fuji Film) that automatically detects differential gene expression between the two arrays (Tables 1, 2). PSL units can be used to quantify results from the BAS system (Fuji Film) The PSL value is proportional to radioactivity × exposure time. Each amount of radioactivity has a different proportionality coefficient with this definition. The ratio of each spot density was determined between the control and after TGF-β1 treatment. Membranes were then exposed to x-ray film for 1 to 3 days at −70°C.

Gene-Specific RT-PCR Analysis

Gene-specific RT-PCR was used to confirm the differential expression of genes identified on the expression array. First-strand cDNA synthesis was performed with a first-strand cDNA synthesis kit (Advantage; Clontech), oligo(dT), and reverse transcriptase (SuperScriptII; Gibco BRL). RT reactions were performed under RNase-free conditions. Briefly, 10 μg total RNA and oligo(dT) was heat denatured at 70°C for 10 minutes and quick chilled on ice before the RT reaction. Afterward, a 25-μl volume containing 5 μl 5× first-strand buffer, 1 μl 10× dNTP, 2.5 μl of dithiothreitol (100 mM), 0.5 μl RNase inhibitor, and 1 μl reverse transcriptase were added to the RNA and incubated for 1 hour at 37°C. The reaction was terminated by incubation at 70°C for 20 minutes. The cDNA synthesis products were used to perform PCR reactions for 20 cycles with the PCR kit (Qiagen, Valencia, CA). Oligonucleotide primers, which were designed based on the GenBank sequences (Table 3; available in the public domain from the National Center for Biotechnology Information, Bethesda, MD, at http://www.ncbi.nlm.nih.gov), were used in RT-PCR reactions.

To determine the linear range of amplification for β-actin, the degree of amplification after each PCR cycle between cycles 20 and 30 was determined. The following protocol was used for the PCR amplification: 2 minutes at 94°C, 24 cycles of 1 minute at 93°C, 1 minute at 62°C, 1 minute at 72°C, and 5 minutes at 72°C. PCR products were analyzed on standardized 1.5% agarose gels and stained with ethidium bromide.

Fluorscein Imaging of RT-PCR Products

We further measured the density of the RT-PCR products of the four significantly downregulated genes (FluorImager system; Molecular Dynamics, Sunnyvale, CA). PCR products were analyzed on standardized 1.5% agarose gels, and stained with fluorescent dye (SYBR Green I; FMC Bioproducts, Rockland, ME). Changes in expression levels of the four genes were detected by the imaging system and were calculated by computer (Image Quant software; Molecular Dynamics).

Western Blot Analysis

HCECs in the presence or absence of TGF-β1 (10 ng/ml) were extracted after 48 hours and solubilized in lysis buffer (50 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 0.15% deoxycholate [wt/vol], 0.1% SDS [wt/vol], 10 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1% NP40 [wt/vol], 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethyl sulfonyl fluoride). The samples were then centrifuged at 15,000*g* for 20 minutes at 4°C. Protein concentrations of the supernatants were measured as previously described,[25] with bovine serum albumin as a standard. Each sample (10 μg) was analyzed both by SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blot analysis, using an anti-integrin α3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-caspase10 polyclonal antibody (Santa Cruz), an anti-insulin-like growth factor I recep-

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TABLE 1. Genes Downregulated Under TGF-β1 Treatment

Gene	Ratio*	Density Control (PSL)	Density TGF-β1 (PSL)	GenBank Accession Number
Integrin α3	0.0824 ± 0.0002	448.64 ± 0.20	36.97 ± 0.05	M59911
PAI-2	0.1239 ± 0.0010	204.31 ± 0.17	25.31 ± 0.22	M18082
Transferrin receptor	0.1246 ± 0.0016	73.41 ± 0.28	9.15 ± 0.09	X 01060
CCND1	0.1303 ± 0.0005	198.78 ± 0.23	25.91 ± 0.11	X 59798
Cadherin1	0.1407 ± 0.0011	89.12 ± 0.19	12.54 ± 0.07	X 13009
40S ribosomal protein S19	0.1410 ± 0.0002	1060.66 ± 0.31	149.51 ± 0.13	M81757
c-Src kinase	0.1643 ± 0.0045	40.43 ± 0.33	6.64 ± 0.13	X59932
Heme oxygenase 2	0.1820 ± 0.0031	56.37 ± 0.26	10.26 ± 0.15	O21243
Hint protein (protein kinase C inhibitor 1)	0.1934 ± 0.0007	464.57 ± 0.09	89.84 ± 0.31	U 51004
HDGF	0.2001 ± 0.0007	203.35 ± 0.33	40.69 ± 0.21	D16431
IL-1α precursor	0.2131 ± 0.0016	223.80 ± 0.21	47.69 ± 0.39	X 02851
Alzheimer's disease amyloid A4protein precursor	0.2135 ± 0.0020	477.19 ± 0.35	101.88 ± 0.14	Y00264
Heat shock protein 40	0.2150 ± 0.0060	281.76 ± 0.19	60.59 ± 0.16	D49547
CTLA3	0.2214 ± 0.0016	128.33 ± 0.10	28.41 ± 0.19	M18737
IL-1R1	0.2222 ± 0.0056	65.16 ± 0.19	14.48 ± 0.34	M27492
BPAG1	0.2229 ± 0.0012	178.01 ± 0.24	39.68 ± 0.16	M63618
RHO12	0.2233 ± 0.0013	1984.91 ± 0.13	443.19 ± 0.22	L25080
TDPX2	0.2247 ± 0.0007	267.05 ± 0.15	59.99 ± 0.20	X67951
Heat shock cognate 71-kDa protein	0.2291 ± 0.0005	238.27 ± 0.11	54.58 ± 0.11	Y00371
CNBP	0.2341 ± 0.0015	263.07 ± 0.09	61.58 ± 0.40	M28372
VEGFR-1	0.2385 ± 0.0003	504.32 ± 0.18	120.26 ± 0.10	X51602
80k-H protein	0.2489 ± 0.0020	241.81 ± 0.31	60.17 ± 0.33	J03075
Receptor eph (ephrin type A receptor I precursor)	0.2502 ± 0.0002	507.17 ± 0.26	126.90 ± 0.15	M18391
p60 Lymphocyte protein	0.2505 ± 0.0005	354.08 ± 0.14	88.71 ± 0.16	M34664
26s Protease regulatory subunit 6A	0.2554 ± 0.0001	291.68 ± 0.41	74.48 ± 0.13	M34079
MTP (Golgi 4-transmembrane spanning transporter)	0.2556 ± 0.0037	63.45 ± 0.27	16.22 ± 0.16	D14696
PGI	0.2561 ± 0.0024	123.61 ± 0.42	31.66 ± 0.22	K03515
RAF-1	0.2598 ± 0.0042	48.95 ± 0.22	12.72 ± 0.15	X03484
P68 kinase	0.2704 ± 0.0015	47.15 ± 0.24	12.75 ± 0.12	M35663
ILF+ILF2+ILF3	0.2751 ± 0.0035	60.27 ± 0.28	16.58 ± 0.13	U58196
14-3-3 protein β	0.2775 ± 0.0010	141.69 ± 0.23	39.32 ± 0.20	X57346
ITGB6	0.2782 ± 0.0011	81.20 ± 0.26	22.59 ± 0.10	M35198
EDDR1	0.2842 ± 0.0012	45.85 ± 0.13	13.03 ± 0.09	X74979

Data are mean ± SD.
* Ratio: TGF-β1/control.

tor β (IGF-IR) polyclonal antibody (Santa Cruz), an anti-transferrin receptor polyclonal antibody (Santa Cruz), and an anti-PAI-2 monoclonal antibody (American Diagnostica, Inc, Greenwich, CT). Horseradish peroxidase–conjugated antibodies were used for the secondary antibodies (1 hour; room temperature). Immunoreactive proteins were visualized on x-ray film using a chemiluminescent protein detection system (Immun-Star; Bio-Rad, Herts, UK).

RESULTS

cDNA Expression Array

To investigate the effects of TGF-β1 on gene expression, we analyzed the cDNA expression arrays using the cDNA probes obtained from either normal or TGF-β1–treated HCECs (Figs. 1A, 1B). No signals were visible in the blank spots and the negative control spots, indicating that the hybridization was highly specific. A comparison of the results of phosphorimaging the two arrays shows that a large number of genes changed their level of expression (Figs. 1A, 1B). To determine reproducibility, a series of the experiments was performed three times using RNA isolated from three separate cultures of either normal or TGF-β1–treated HCECs (six hybridizations to six cDNA array membranes). The results were remarkably similar, and the intensity readings for each gene were averaged from the three experiments to obtain the values listed (Tables 1, 2). Figure 1C shows the bivariate log–log scatterplot of the mean values of 1176 genes obtained from three independent series of hybridizations of cDNA array in the absence or presence of

TABLE 2. Genes Upregulated Under TGF-β1 Treatment

Gene	Ratio*	Density Control (PSL)	Density TGF-β1 (PSL)	GenBank Accession Number
Ras-related protein RAB-7	3.3629 ± 0.0090	22.79 ± 0.11	76.64 ± 0.17	X93499
SCYA5	3.2591 ± 0.0066	72.18 ± 0.19	235.26 ± 0.16	M21121
ITGB4	3.1786 ± 0.0063	127.83 ± 0.19	406.34 ± 0.22	X53587
ICE-LAP4 (caspase 10)	3.1419 ± 0.0057	63.25 ± 0.07	198.72 ± 0.15	U60519
L-CA	3.0903 ± 0.0089	7.25 ± 0.10	22.41 ± 0.24	Y00638
Cyclin K	3.0838 ± 0.0095	26.22 ± 0.07	80.85 ± 0.12	AF060515
ITGAL	3.0323 ± 0.0020	134.04 ± 0.09	406.46 ± 0.04	Y00796

Data are mean ± SD.
* Ratio: TGFβ1/control.

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Table 3. Oligonucleotide Primers for RT-PCR

PCR Primers	Product Size (bp)	GenBank Accession Number
β-Actin		
5′-CATGAAGTGTGACGTGGACATC-3′		
5′-CGGACTCGTCATACTCCTGCTT-3	251	X00351
Downregulated genes		
Integrin α3		
5′-TGCTGTATCCCACGGAGATCAC-3′		
5′-CTGTTCCACACTCGTGCCTTCA-3′	319	M59911
PAI-2		
5′-GCTTCCAGATGAAATTGCCGA-3′		
5′-GCTTCAGTGCCCTCCTCATTC-3′	306	M18082
TFRC		
5′-TGGCTGTATTCTGCTCGTGGA-3′		
5′-CAATGTCCCAAACGTCACCAG-3′	349	X01060
HPRAD1		
5′-AGAACACGGCTCACGCTTACCT-3′		
5′-GCTGGAAACATGCCGGTTACAT-3	284	X59798
Upregulated genes		
Ras-related protein RAB-7		
5′-CTGGTATTTGATGTGACTGCCC-3′		
5′-GGAAATTCGTTGTACAGCTCCA-3′	305	X93499
ITGB4		
5′-GGTCCAGGAAGATCCATTTCAA-3′		
5′-TAGCAGACCTCGTAGGCTGTGA-3′	355	X53587
Caspase10		
5′-GGTTGGAACATTTCAGTTGCCA-3′		
5′-TCAAGGCGTCCTTACAGAGCCA-3′	373	U60519
Steady genes		
TSE		
5′-TCTGCCTCGGCTCACAAAT-3′		
5′-ACTTTGTCCGCCTGGCATT-3′	284	M33336
Caspase3		
5′-GTGCTATTGTGAGGCGGTTGT-3′		
5′-TTCCAGAGTCCATTGATTCGC-3′	299	U13737
IGF-IR		
5′-AGAAGGAGGAGGCTGAATACCG-3′		
5′-GTTGCAGCTGTGGATATCGATG-3′	287	X04434

TGF-β1. In the system of the cDNA expression array (Atlas; Clontech), a change greater than twofold in signal intensity is reported to be a significant difference.[26] In accordance with this criterion, we analyzed the effect of TGF-β1 on the gene expression levels.

Of the 1176 genes analyzed, 277 genes showed a decrease of expression in HCECs on TGF-β1 treatment. In Table 1, we list the genes that showed a greater than 3.5-fold change. These genes have been listed according to the TGF-β1-to-control densitometric ratio. For example, the expression of α3-integrin decreased the most (12.1-fold), followed by PAI-2 (8.1-fold), transferrin (8.0-fold) and cyclin-D1 (7.7-fold). By contrast, the expression of only 19 genes was increased by TGF-β1 treatment. In Table 2, we list the genes whose change in signal intensity was greater than threefold.

To confirm this gene array analysis, we performed relative RT-PCR (Fig. 2). We analyzed the expression of the four genes that, in response to TGF-β1 treatment, showed the largest decrease in expression with the human cDNA expression arrays. These four genes were also found to be diminished by gene-specific RT-PCR (Fig. 2, top). Strong bands were detected in the HCEC control samples, but only very faint bands were observed on TGF-β1–treated samples (Fig. 2, top). TGF-β1 treatment did not change the intensity of β-actin fragments (control experiment; Fig. 2, second from top). We further analyzed the expression of three genes that were increased and three genes that were not affected by TGF-β1 treatment. Consistent with the cDNA array analysis, the RT-PCR products of the three genes upregulated by TGF-β1 treatment were enhanced significantly. We could observe no change in the levels of the three unaffected genes. The densities of the RT-PCR products of the four downregulated genes were further ana-

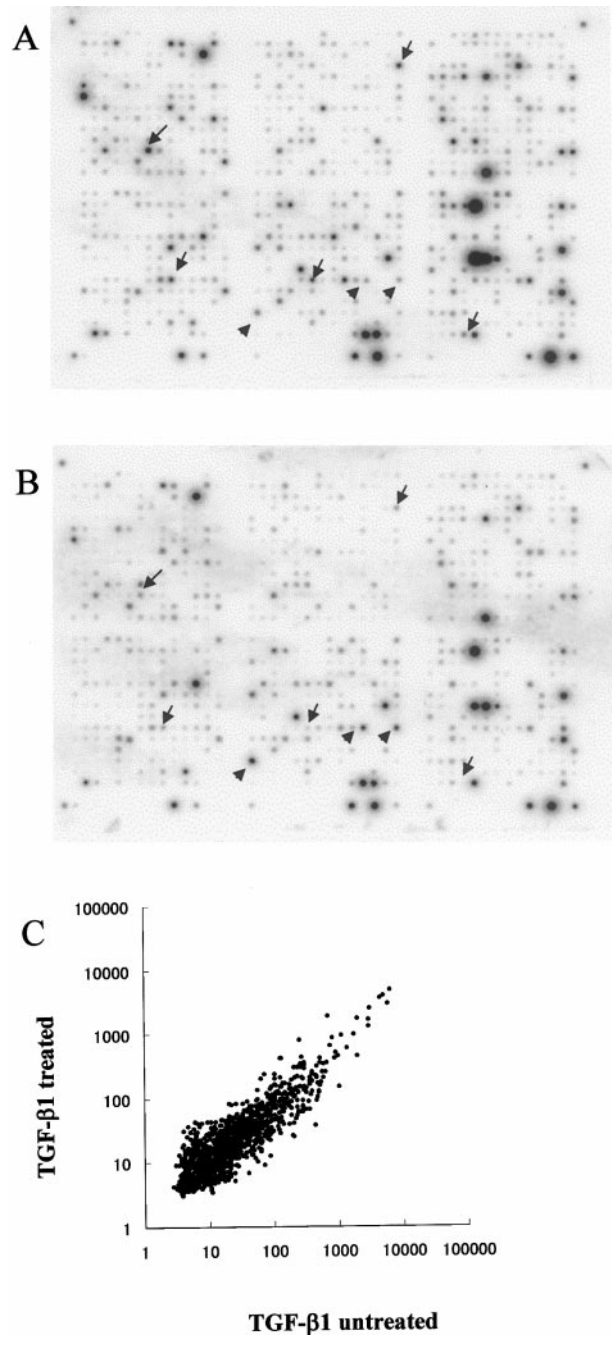

Figure 1. Parallel analysis of gene expression between control HCECs (**A**) and TGF-β–treated HCECs (**B**) using the cDNA expression array. Labeled cDNA was synthesized from total RNA isolated from both cell populations and hybridized to the cDNA array blots. The change of the expression levels of various genes between the two was probed. The intensity of the hybridized signals was measured by densitometry scanning. *Arrows*: representative cDNA spots that show significant decreases in expression. *Arrowheads:* spots of increased cDNA expression. (**C**) Scatterplot matrix of densitometric quantitation of expression data from TGF-β1–treated or untreated HCECs for 1176 genes.

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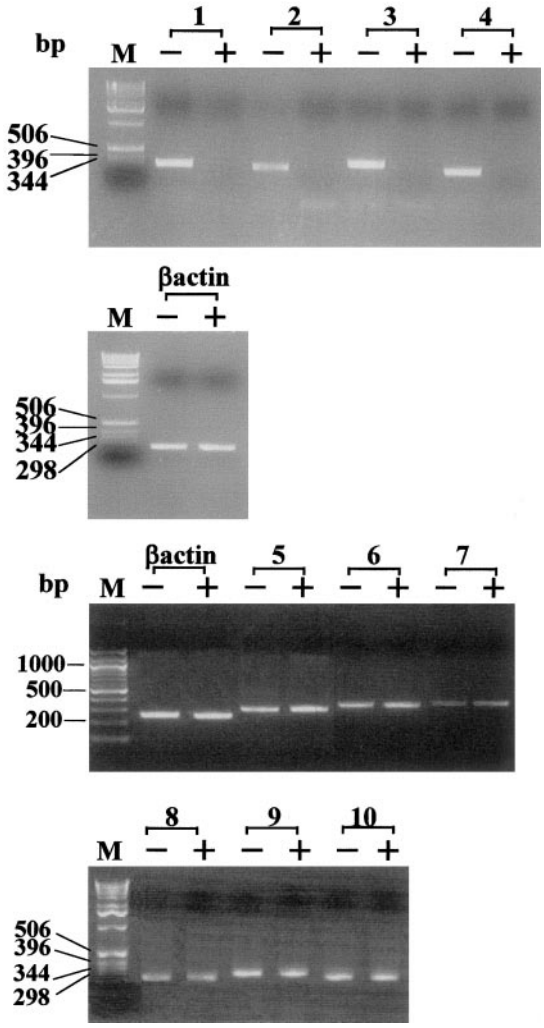

FIGURE 2. RT-PCR analysis of mRNA from control HCECs and TGF-β1–treated HCECs. The target genes were selected on the basis of the results obtained from the cDNA arrays. PCR products were analyzed on 1.5% agarose gels and subsequently stained with ethidium bromide. The sets of primers specific for α3-integrin (*lane 1*), PAI-2 (*lane 2*), transferrin receptor (*lane 3*), cyclin-D1 (*lane 4*), RAB-7 protein (*lane 5*), integrin beta4 (*lane 6*), ICE-LAP4 (caspase10 precursor; *lane 7*), TSE1 (*lane 8*), Caspase3 (*lane 9*), IGF-IR (*lane 10*), and β-actin (control experiment, the second and third panels from the *top*) were used. (−) untreated; (+) TGF-β1–treated.

lyzed by fluorescein-imaging (FluorImager; Molecular Dynamics; data not shown).

To determine the linear range of amplification, we observed the degree of amplification of β-actin after each PCR cycle between cycles 20 and 30. Using this standard, we normalized and compared the data for 10 genes. The RT-PCR and fluorescein-imaging analyses were performed three times using RNA isolated from cultures of either normal or TGF-β1–treated HCECs. The densitometric ratio of TGF-β1 to control (means ± SD) is shown in Table 4. The TGF-β1-to-control ratio of each of the four genes obtained from the array system was very close to that obtained from RT-PCR.

Western Blot Analysis

To detect the change of gene expression induced by TGF-β1 at the protein level, we used Western blot analysis. Based on the cDNA expression array analysis, PAI-2, transferrin receptor, and integrin α3 were investigated as representative genes downregulated by TGF-β1. We also studied two unaffected genes, IGF-IR and Caspase3, and an upregulated gene, ICE-like apoptotic protease 4 (caspase10). Immunoblot analysis clearly showed that the bands for the PAI-2, transferrin receptor, and integrin α3 proteins were significantly diminished on TGF-β1 treatment (Fig. 3A). The results of the expression changes at the protein level were consistent with those from the cDNA array and RT-PCR analysis. Moreover, the intensity of IGF-IR and Caspase3 fragments were not affected by TGF-β1 (Fig. 3B), and the immunoblot-band intensity of caspase10 was enhanced by TGF-β1 (Fig. 3C). These observations are also consistent with cDNA expression. These results clearly indicate that TGF-β1 can regulate the expression of specific molecules at the protein level.

DISCUSSION

The major findings of this study are that TGF-β1 regulated the expression of specific genes expressed in HCECs and that the number of genes downregulated by TGF-β1 treatment was much larger than that of the genes upregulated. The latter finding was rather surprising, because TGF-β1 has been suggested to increase gene expression levels.[27] Therefore, we emphasize in this report the molecules whose expression was downregulated by TGF-β1. Among the 277 genes that were downregulated by TGF-β1, α3-integrin, the transferrin receptor, cyclin-D1, and PAI-2 showed the largest decrease. These four genes have been reported to exist in the corneal epithelium.[26,28–30] To our knowledge, this is the first report to identify the specific genes downregulated by TGF-β1 in the corneal epithelium.

PAI-2 is a member of the family of serine protease inhibitors and has been proposed to be involved in cellular changes associated with pregnancy, inflammation, apoptosis, and cell differentiation.[31–33] It has also been shown to play a key role in the differentiation of epidermal keratinocytes.[34,35] PAI-2 expression in epidermal epithelial layers is strongly enhanced in the final stage of terminal differentiation[32,33] However, functional involvement of PAI-2 expression in the corneal epithelium has been unclear. This study has demonstrated for the first time that TGF-β1 causes a dramatic decrease in the expression of PAI-2 in the corneal epithelium. Although PAI-2 is expressed in all cell layers of the normal human corneal epithelium, the expression is especially concentrated in the most superficial cell layers.[29] Because TGF-β receptors are expressed much more strongly in the basal cell layers than in the superficial cell layers of the corneal epithelium,[7,17] the expression pattern of PAI-2 may be dynamically controlled by TGF-β1 and therefore should be detected in the more superficial cell layers. Previous studies have demonstrated that TGF-β1 may inhibit differentiation of several epithelial cells, including those in the corneal epithelium.[1,2,35,36] We hypothesize that TGF-β1 may cause inhibition of corneal epithelial differentiation through a decrease in expression of PAI-2. Further studies are required to

TABLE 4. Comparison of Data from Fluoimaging and cDNA Arrays

Gene	Fluoimaging Density Ratio TGF-β1/Control	cDNA Expression Array Density Ratio TGF-β1/Control
Integrin α3	8.3 ± 0.11	8.2 ± 0.02
PAI-2	11.1 ± 0.15	12.4 ± 0.10
Transferrin receptor	12.1 ± 0.10	12.5 ± 0.16
Cyclin D1	12.5 ± 0.19	13.0 ± 0.05

Data are mean percentages ± SD.

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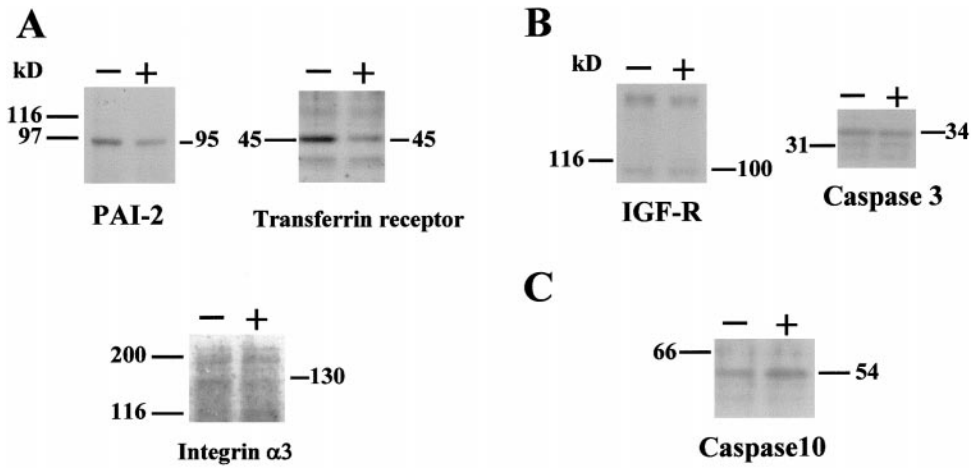

FIGURE 3. Biochemical analysis of the effect of TGF-β1 on the expression of various proteins expressed in HCECs. Each protein (10 μg) extracted from HCECs in the presence or absence of TGF-β1 (10 ng/ml) was applied to the gel and analyzed by antibodies specific to the downregulated genes integrin α3, PAI-2, and transferrin receptor (**A**); the steady genes, IGF-IR and caspase3 (**B**); and the upregulated gene, caspase10 (**C**), after TGF-β1 treatment.

understand the function of PAI-2 regulation in the corneal epithelium.

Transferrin acts as a growth factor in corneal epithelial cells, as well as in hepatocytes and cells in other types of epithelia.[37,38] The transferrin receptor is known to be expressed in HCECs.[30,39] It is located at the cell surface and is present in increased amounts on the surface of proliferating cells during cell division.[40] The present results clearly showed that TGF-β1 decreased gene expression of the transferrin receptor in the corneal epithelium. TGF-β1 has been reported to inhibit corneal epithelial proliferation.[1,2] Therefore, it seems probable that TGF-β1 acts to block corneal epithelial proliferation through the inhibition of transferrin receptor expression. It would be interesting to test whether TGF-β1 acts similarly in vivo in human corneal epithelium. The mechanism for the downregulation of transferrin receptor expression by TGF-β1 is unclear, and thus further investigation is needed.

The integrin gene superfamily plays a major role in the mediation of adhesive interactions between cells and their scaffolds. Integrins also facilitate the migration of epithelial cells.[31] Functional integrin in vivo is composed of a heteromultimer of α and β subunits. In corneal epithelial cells, a specific combination of α3 and β1 subunits is expressed. The expression of α3 and β1 integrins has been shown to be most intense in the basal layer that is attached to the basement membrane, and because this integrin complex has a strong affinity for laminin and collagen, which constitute the basement membrane, this complex may be crucial for the adhesion of basal cells to the basement membrane in the cornea.[41] Downregulation in α3-integrin expression induced by TGF-β1 has been reported in MG-63 human osteosarcoma cells,[42] and our report demonstrates that the same phenomenon occurred in the corneal epithelium. Other studies have demonstrated contrary results showing that TGF-β1 can facilitate cell adhesion by increasing the expression and deposition of extracellular matrix proteins and by upregulating the expression of integrins.[43–46] Nevertheless, in accordance with our results, it seems probable that the disappearance of basal cell adhesion results from a decrease in integrin α3 protein expression induced by TGF-β1, which, in turn, causes a collapse of the cell–cell network. It is premature to speculate whether similar events occur in other cell types or conditions. However, the involvement of TGF-β1 in cell adhesion and cell differentiation in the corneal epithelium is an attractive theme for further investigation.

Cyclin-D1, -D2, and -D3 are key molecules that regulate cell cycle progression. Cyclin-D1 is specifically expressed during the G1 phase and induces the S phase.[47,48] TGF-β1 can inhibit cell proliferation by arresting cells at the G1- to S-phase transition.[49] This phenomenon has been proposed to occur through the inhibition of cyclin-dependent kinases (cdks), molecules responsible for cell cycle progression. The induction of p21 and p15 by TGF-βs inactivates the catalytic activity of cdks and prevents the assembly of new cyclin D-cdk complexes from latent pools.[28] In this study, we clearly demonstrate that TGF-β1 specifically decreases cyclin-D1 expression in HCECs. The direct downregulation of cyclin D1 by TGF-β1 should therefore be incorporated into proposed mechanisms for TGF-β1–mediated growth arrest of the corneal epithelium.

Unexpectedly, only a small number of genes, 19 of the 1176 probed, were found to be upregulated after TGF-β1 treatment (Table 2). Among these genes, β4-integrin has been reported to be upregulated by TGF-β1.[50] We have shown in the present study that the expression of 18 other genes, such as ras-related protein RAB-7, small inducible cytokine A5, and caspase10, are induced by TGF-β1. These observations should spawn further investigation of the role of TGF-βs in cell signal transductions.

In summary, we used the cDNA array technique to monitor the change in the overall profile of gene expression in HCECs induced by TGF-β1 treatment. We found that TGF-β1 may control the differentiation and proliferation of corneal epithelial cells through changed expression levels of specific genes. The inhibitory reactions to TGF-β1 by epithelial cells may result from the simultaneous downregulation of a variety of molecules, including PAI-2, transferrin, integrin α3, and cyclin-D1. Our results thus reinforce the physiological significance of TGF-β1. TGF-β1 can therefore regulate the corneal epithelium by changes in the network of various signal-transduction pathways. This combined strategy of cDNA expression array, RT-PCR, and Western blot analysis provides a novel approach to clarifying the effects of TGF-β1 on the corneal epithelium.

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