Microarray Analysis of Corneal Fibroblast Gene Expression after Interleukin-1 Treatment

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Purpose. To identify changes in gene expression in human corneal fibroblasts after exposure to interleukin-1α.

Methods. RNA was isolated from cultured human corneal fibroblasts after treatment with interleukin-1α and subjected to DNA microarray analysis. Changes in gene expression were determined by comparison with untreated cells in three independent experiments after a Bayesian statistical analysis of variance.

Results. Changes in gene expression were reproducibly observed in 165 genes representing previously identified and novel chemokines, matrix molecules, membrane receptors, angiogenic mediators, and transcription factors that correlated with pathophysiological responses to inflammation. Dramatic increases in gene expression were observed with exodus-1 (CCL20), MMP-12, and RhoA.

Conclusions. DNA microarray analysis of the corneal fibroblast response to interleukin-1α provides important insight into modeling changes in gene expression and suggests novel therapeutic targets for the control of corneal inflammation. (*Invest Ophthalmol Vis Sci.* 2002;43:2143–2151)

The cornea is a transparent, five-layered tissue that serves as a critical barrier to ocular infection and injury. The stromal layer constitutes 90% of the corneal thickness and is a frequent site of inflammation after infection, trauma, or surgery. Although the inflammatory response may initially help control an infection or seal a wound, all too often the process leads to edema, collagen disorganization, neovascularization, ulceration, and scarring. Current anti-inflammatory treatment is frequently inadequate to prevent destruction of tissue and loss of corneal transparency.[1,2] In severe cases, the only option is corneal transplantation, which is itself subject to failure after excessive inflammation. Thus, uncontrolled inflammation within the cornea may significantly decrease visual acuity or end in blindness.[3] Understanding the endogenous inflammatory response is critical for developing improved treatment options for the more than 40,000 patients annually who undergo corneal transplantation because of cornea-related blindness.[4]

Interleukin-(IL)-1α is an important mediator of early inflammation in the cornea.[5–9] The cytokine IL-1α is produced early by epithelial cells and resident corneal fibroblasts and later by invading leukocytes.[7,10] Expression of IL-1α is upregulated after corneal grafting,[11] infection,[12] epithelial trauma,[6] and alkali burns.[13] As one of the earliest cytokines released into the corneal stroma, IL-1α amplifies the inflammatory response through membrane receptors expressed by fibroblasts.[7,14] During dynamic phenotypic changes, these cells alter their shape, become motile, elaborate additional inflammatory factors, and regulate stromal matrix components.[8,15,16] This response is in part due to altered gene expression mediated by IL-1α, yet only a limited number of these genes are known.

With the advent of gene therapy and the increased number of small-molecule pharmaceuticals, it is increasingly important to discover which genes might serve as targets in controlling excessive inflammation. DNA microarrays offer a powerful method to identify potential targets by simultaneously screening expression changes in thousands of genes.[17] In a culture model of corneal inflammation, we applied this technique to monitor early changes in gene expression in corneal fibroblasts exposed to IL-1α. Using novel statistical software, a model for genes involved in corneal inflammation was developed with the hope that this approach may eventually identify specific therapeutic avenues for the reduction of corneal opacification.

Materials and Methods

Cells Culture and RNA Isolation

Cell culture reagents were purchased form Gibco-BRL (Grand Island, NY) and IL-1α from Calbiochem (La Jolla, CA). A fresh human cornea was obtained from the Lions Doheney Eye Bank (Los Angeles, CA). A central section measuring 6 mm in diameter was excised, and epithelial and endothelial layers were scraped away. The remaining stromal layer was minced, and explants were cultured in Eagle's minimum essential medium with 20% fetal bovine serum, 100 IU penicillin G, and 100 μg/mL streptomycin in 35-mm dishes (Corning, Inc., Corning, NY). After 14 days, corneal fibroblasts became confluent and were subcultured. Fifth-passage corneal fibroblasts were then grown on 100-mm dishes in DMEM supplemented with 10% fetal bovine serum until 80% to 90% confluent. Cells were then rinsed and cultured in cell culture medium (Opti-MEM; Gibco) for an additional 72 hours, as previously described.[18] Corneal fibroblasts were treated for 24 hours with either vehicle (EtOH) or IL-1α (10 ng/mL) in three independent experiments. Afterward, cells were trypsinized, and approximately 20 μg total RNA was isolated with an RNA extraction system (RNeasy; Qiagen, Valencia, CA), according to the manufacturer's protocol. The RNA concentration was determined by spectroscopy, and the quality was confirmed by gel electrophoresis.

cRNA Preparation and Array Hybridization

Total RNA was converted into double-stranded cDNA using a custom kit (SuperScript II Double-Stranded cDNA Synthesis Kit; Life Technologies, Gaithersburg, MD). After ethanol extraction, in vitro transcription reactions were performed (BioArray HighYield RNA Transcript Labeling Kit; Enzo Biochemicals, Inc., Farmingdale, NY) according to the manufacturer's protocol. Purified, labeled cRNA was quantified by spectrophotometric analysis, qualitatively analyzed for size distribution by gel electrophoresis, and fragmented to 30 to 60 base fragments with Tris-acetate (pH 8.1; 40 mM), KOAc (100 mM), and MgOAc (30 mM) in

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a 20-μL volume heated for 35 minutes at 94°C. Protocols and instrumentation setups, including total RNA samples, hybridization to U95A human microarrys, washing, staining, and scanning were followed as recommended in the manufacturer's technical manual (GeneChip Microarrays; Affymetrix, Santa Clara, CA).

Data Analysis

The resultant arrays were scanned twice in the system's confocal scanner (HP GeneArray Scanner; Hewlett Packard, Palo Alto, CA). Data analysis was first performed with the software accompanying the microarrays (GeneChip Expression Analysis Software, ver. 3.3; Affymetrix) to obtain average difference intensities. The Cyber-T software package developed at the University of California, Irvine, California, (provided in the public domain by the National Center for Genome Resources, Santa Fe, NM, at http://genomics.biochem.uci.edu/genex/cybert/) was then used to determine average intensity values of genes across three independent experiments and to compare values for control- and IL-1α–treated cells. This list was further restricted based on whether genes were consistently present or consistently absent in three independent experiments. By using a Bayesian statistical analysis, significant changes were ranked by the assignment of probabilities.[19] Genes displaying intensities lower than 30 were empirically considered absent. Multiples of changes in gene expression were calculated by comparing average intensities in control with IL-1α treatment. For genes that were entirely absent in either control or IL-1α treatment but present in the other condition, relative multiples of change were assigned by comparing intensities with a normalized value determined by the software (Affymetrix).

Results

The U95A human gene microarray is composed of oligonucleotides representing approximately 12,000 full-length, partially sequenced, and expressed sequence tag genes. Of the sequences represented, approximately 7700 were detected in cRNA produced from cultured human corneal fibroblasts, and 3532 of these correlated with complete cDNAs. Pair-wise comparison of gene expression levels in three independent control experiments displayed an overall correlation coefficient of $R^2 = 0.95$, suggesting excellent reproducibility (data not shown).

Corneal fibroblasts were treated with IL-1α for 24 hours, as in previous studies,[18] and cRNA was generated for microarray analysis. A common approach to identifying significant changes in gene expression is based on selection by multiples of change. After IL-1α treatment, 1115 genes displayed a greater than threefold expression change (data not shown) on average. Yet, many of these were changes with high interexperimental variance and probably represented experimental artifacts. An alternative approach is based on applying the *t*-test within a Bayesian statistical framework and using probabilities rather than multiples of change to rank significant expression changes. This method was preferred, because it more conservatively identifies genes with expression changes and reduces false-positive findings in microarray studies, with few replicates.[19,20]

After statistical analysis, significant changes in messenger RNA levels were detected in only 14 genes with $P < 0.01$ (Table 1, asterisks). Expanding the selection of genes to those with a $P < 0.05$ generated an additional 118 genes (Table 1). Some of the genes were especially noteworthy for their inflammatory roles and are described in the results (Table 1). In addition, 22 genes absent in untreated cells were detected after IL-1α treatment (Table 2), and 25 genes became undetectable (Table 3). Several unknown or incomplete sequences were also identified. This included 55 sequences with a $P < 0.05$: 6 detected only after IL-1α treatment and 16 that became undetectable after IL-1α treatment (data not shown). Altogether, these results suggest that the number of highly reproducible changes in gene expression were limited to approximately 2% of the sequences sampled. This is in spite of the extremely sensitive detection system and application of a highly potent cytokine.

Identification of Control Genes

Studies demonstrate that gene detection by microarrays correlates with standard methods such as Northern analysis and RT-PCR.[21] In untreated corneal fibroblasts, for example, the microarray experiment detected 521 corneal genes previously catalogued by various methods.[22] At the same time, genes specifically not expressed by passaged corneal fibroblasts, such as the type 2 IL-1 receptor, collagenase, and stromelysin were appropriately absent.[23,24] To further confirm whether previously described changes in gene expression in IL-1α–treated corneal fibroblasts were evident in these microarray studies, we examined genes for IL-1α, regulated on activation normal T-cell expressed and secreted (RANTES), and granulocyte-macrophage–colony-stimulating factor (GM-CSF)-1.[18,25] These genes served as internal controls, and, consistent with previous studies, each of these genes was upregulated. In treated cells IL-1α gene expression increased sixfold, RANTES eightfold, and GM-CSF-1 sevenfold (Tables 1, 2). These findings lend strong support to the statistical methods used for gene selection and the validity of the microarray analysis within the biological context. Some IL-1α–responsive genes such as hepatocyte growth factor,[26] monocyte chemotactic protein-1,[18] and GRO-α,[27] were not represented on the microarray and could not be tested.

IL-1α–responsive genes have also been studied in cells of different tissue origin. The microarray analysis identified similar expression changes not previously described in the corneal fibroblasts. Some of these were the modulation of interferon-β2, endothelin-1, platelet-derived growth factor (PDGF)-α receptor, thrombospondin, manganese superoxide dismutase, Gro-β, Gro-γ, plasminogen activator-inhibitor (PAI) 2, c-*myc*, and IL-6.[10] No expression changes were observed in inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, tissue inhibitor of matrix metalloproteinase (TIMP)-1, metalloproteinase (MMP)-1, IL-8, or keratinocyte growth factor. This may reflect either a tissue-specific expression or response, or in the case corneal fibroblast genes, differences in the experimental conditions.[24,28]

Secreted Inflammatory Genes

As one of the earliest expressed cytokines, IL-1α propagates the expression of additional cytokines that amplify the inflammatory process. Genes for both IL-1α and IL-1β were upregulated (Table 1, 2). It is important to note that the increased IL-1β RNA detected may reflect increased stabilization of its mRNA rather than increased transcription and that some IL-1–responsive genes, such as keratinocyte growth factor, may depend specifically on the IL-1β isoform.[26,29] Interferon-β2a, which is identical with IL-6, was upregulated and may have a protective role during corneal infection.[30] Interferon-α, which displays antiviral, antiparasitic, and antiproliferative activities,[31] was downregulated. This could create a permissive environment for fibroblast mitogenesis and viral infection.

Chemokines are cytokines that recruit leukocytes to sites of inflammation.[32] In response to IL-1α, for example, corneal fibroblasts upregulate and secrete MCP-1 (CCL2), RANTES (CCL5), and GROα (CXCL1).[6,18] The microarray experiments identified two other growth-regulated oncogenes (GRO) genes: GRO-β (XXCL2) and GRO-γ (CXCL3). The chemokine exodus-1/MIP-3α/LARC (CCL20),[33,34] displayed a dramatic 29-fold increase in expression. Monocyte chemotactic protein-2

TABLE 1. Genes Expression Changes after IL-1α Treatment

Accession Number	Gene Description	Change (×)
Extracellular		
Inflammation		
U64197	Exodus-1*	29.2
Y16645	Monocyte chemotactic protein-2	14.8
M21121	T-cell–specific protein (RANTES)*	8.3
M13207	GM-CSF-1	7.2
M15330	Interleukin-1-beta (IL-1β)	6.5
M36821	GRO-gamma*	4.1
X04430	IFN-beta 2a (IL-6)	3.9
M36820	GRO-beta	3.2
X78686	ENA-78	3.1
Matrix		
L23808	Human metalloproteinase (HME) MMP-12*	10.4
J05008	Homo sapiens endothelin-1 (EDN1)*	8.8
Y00630	Plasminogen activator-inhibitor 2, (PAI-2)	7.7
X14787	Thrombospondin	4.1
L02870	Alpha-1 type VII collagen (COL7A1)	3.5
U76702	Follistatin-related protein (FLRG)	3.2
M60278	Heparin-binding EGF-like growth factor	2.6
M14113	Coagulation factor VIII-C	−2.7
M58526	Alpha-5 collagen type IV (COL4A5)	−3.2
X78947	Connective tissue growth factor	−3.2
X15998	Chondroilin sulphate proteoglycan versican, V1 splice-variant	−3.4
K02054	HUMGRP5E Human gastrin-releasing peptide	−3.8
AB011538	MEGF5*	−4.1
S78569	Laminin alpha 4 chain*	−5.4
Membrane Protein		
S46950	Adenosine A2 receptor	9.7
U55258	hBRAVO/Nr-CAM precursor	9.1
M57230	Membrane glycoprotein gp130	5.4
U08023	Cellular proto-oncogene (c-mer)	5.1
Z48199	Syndecan-1 gene (exons 2-5)	4.7
S77812	Vascular endothelial growth factor receptor/ VEGF receptor/cell surface tyrosine kinase	4.7
M23263	Androgen receptor (HUMARB)	4.3
AF035121	KDR/flk-1 (VEGF receptor)	4.0
AL022314	Novel trypsin family protein with class A LDL receptor domains	3.6
L10126	Serine/threonine kinase receptor-2-3 (SKR2-3)	3.5
U77643	K12 protein precursor	3.4
U67784	Orphan G-protein–coupled receptor (RDC1)	3.4
U31628	Interleukin-15 receptor alpha chain precursor (IL15RA)	3.2
X76079	Platelet-derived growth factor alpha receptor	3.1
AF057169	Bestrophin (VMD2), alternatively spliced product	3.1
U97145	RET ligand 2 (RETL2)	3.0
M29696	Interleukin-7 receptor (IL-7)	2.9
U50136	Leukotriene C4 synthase (LTC4S)	2.3
U07695	Human tyrosine kinase (HTK)	−2.5
AF009425	C18orf1, alternative splicing variant alpha-2	−2.7
L06328	Voltage-dependent anion channel isoform 2 (VDAC)	−2.8
L07594	Transforming growth factor-beta type III receptor	−2.8
AF070594	HNK-1 sulfotransferase	−2.8
U81375	hENT1	−2.9
S59184	Related to receptor tyrosine kinase	−3.0
U32324	Human interleukin-11 receptor alpha chain	−3.5
D50406	RECK	−3.9
AL035081	Similar to *Xenopus laevis* mRNA for KDEL receptor	−4.5
Cytosolic		
Structural		
X68742	Integrin, alpha subunit*	7.0
X90761	hHa2 (keratin)	3.4
L43821	Enhancer of filamentation (HEF1)	2.9
M22299	T-plastin	−2.2
AF035812	Dynein light intermediate chain 2 (LIC2)	−2.3
X53002	Integrin beta-5 subunit	−2.5
S67247	Smooth muscle myosin heavy chain isoform SMemb	−2.6
X94232	RP1	−2.6
U97067	Alpha-catenin-like protein	−2.6
U49957	LIM protein (LPP)*	−4.3
Signaling		
U19261	Epstein-Barr virus–induced protein (TNF signaling)	6.2
X68277	CL 100 protein tyrosine phosphatase	4.4
U77735	Pim-2 protooncogene homolog (pim-2h)	3.7
X79780	YPT3	3.2
AF041434	Potentially prenylated protein tyrosine phosphatase hPRL-3	2.5
U50062	RIP protein kinase	2.4
M29893	Low-molecular-mass GTP-binding protein (ral)	−2.1
L33881	Human protein kinase C iota isoform	−2.8
AF039945	Synaptojanin 2B	−3.0
U37352	Phosphatase 2A B′ alpha1 regulatory subunit	−3.4
Y13493	Protein kinase Dyrk2	−3.7
L20321	Serine/threonine kinase stk2*	−3.9
M59371	Human protein tyrosine kinase*	−5.4
Metabolic		
M20681	Glucose transporter-like protein-III (GLUT3)	−3.1
M81118	Alcohol dehydrogenase chi polypeptide (ADH5)	−2.3
U46689	Microsomal aldehyde dehydrogenase (ALD10)	−2.3
AF025887	Glutathione *S*-transferase A4-4 (GSTA4)	−2.9
U37100	Aldose reductase–like peptide	−2.8
X13589	Aromatase (estrogen synthetase)	3.3
X85019	UDP-GalNAc-polypeptide N-acetylgalactosaminyltransferase (T2)	3.1
AF020543	Palmitoyl-protein thioesterase-2 (PPT2)	2.6
D86181	Galactocerebrosidase	−2.5
S69189	Peroxisomal acyl-coenzyme A oxidase	−2.5
M76665	11-beta-Hydroxysteroid dehydrogenase (HSD11)	2.5
AF029893	i-beta-1,3-N-Acetylglucosaminyltransferase	−2.4
U43944	Cytosolic NADP(+)-dependent malic enzyme	−2.8
L29254	(clone P1–5) L-Iditol-2 dehydrogenase	−2.7
L46590	Very long chain acyl-CoA dehydrogenase	2.9
Miscellaneous		
AL022165	Chondroitin 6-sulfotransferase-like protein	7.1
D29992	Placental protein 5 (PP5)	6.9
M92357	B94	3.8
AJ225089	2–5′ Oligoadenylate synthelase 59 kDa isoform	3.6
X54162	64 Kd autoantigen expressed in thyroid and extra-ocular muscle	3.3
AF048730	Cyclin T1	3.0
J02611	Apolipoprotein D	2.8
X07834	Manganese superoxide dismutase	2.6
U11313	Sterol carrier protein-X/sterol carrier protein-2 (SCP-X/SCP-2)	−2.2
M15796	Human cyclin protein gene	−2.2
AJ001625	Pex3	−2.4
AL022326	Synaptogyrin 1A (SYNGR1A)	−2.4
U68723	Checkpoint suppressor 1	−2.4
S74728	Antiquitin=26g turgor protein homologue	−2.5
U70063	Human acid ceramidase	−2.5
D64109	Tob family (Tob2)	−2.5
D83004	Ubiquitin-conjugating enzyme E2	−2.6
AF070523	JWA	−2.6
AB023421	Heat shock protein apg-1	−2.7
U92436	MMAC1 (tumor supressor)	−2.8
AF006010	Progestin-induced protein (DD5)	−2.9
M13452	Lamin A*	−3.2
X82554	SPHAR gene for cyclin-related protein	−3.5
M32886	Sorcin CP-22	−3.8
AF005081	Skin-specific protein (xp320)	−4.2
Nuclear		
U88964	HEM45	8.4
D21205	Estrogen-responsive finger protein	6.4
M14660	ISG-54K gene (interferon-stimulated gene)	4.4
L02932	Peroxisome proliferator–activated receptor	3.9
L78440	STAT4	3.8
X63759	hTNP2	3.7
AB015332	HRIHFB2018	3.4
U51096	Cdx2	3.1
V00568	c-Myc oncogene	3.3
M97935	Transcription factor ISGF-3	3.9
U19969	Two-handed zinc finger protein ZEB mRNA	−2.3
AF008442	RNA polymerase I subunit hRPA39 mRNA	−2.5
AF020043	Chromosome-associated polypeptide (HCAP)	−2.6
AJ223321	RP58 gene	−2.7
V01512	Cellular oncogene c-fos	−2.7
AL031670	Similar to Zinc finger, C3HC4 type (RING finger)	−2.8
AB006909	A-type microphthalmia associated transcription factor	−2.9
AF054284	Spliceosomal protein SAP 155	−3.0
U10686	MAGE-11 antigen (MAGE11)	−3.2
D13666	Osteoblast-specific factor 2 (OSF-2os)*	−3.6
M97815	Cellular Retinoic acid-binding protein II (CRABP-II)*	−5.0
Mitochondrial		
X69433	Mitochondrial isocitrate dehydrogenase (NADP)	−2.9
Y14494	Mitochondrial carrier protein ARALAR1	−3.2
U22028	Human cytochrome P450 (CYP2A13)	−3.6

TABLE 2. Genes Expressed after IL-1α Treatment

Accession Number	Gene Description	Change (×)
Extracellular		
Inflammation		
M28983	Interleukin 1 alpha	6.0
Matrix		
U19557	Squamous cell carcinoma antigen 2 (SCCA2)	9.2
Membrane Protein		
M81830	Somatostatin receptor isoform 2 (SSTR2)	9.9
U10485	Lymphoid-restricted membrane protein (Jaw1)	9.7
L02750	Potassium channel	4.6
Cytosolic		
Structural		
U89330	Microtubule-associated protein 2 (MAP2), alternatively spliced	12.7
AJ005694	Short form of beta II spectrin	10.2
Signaling Proteins		
M12174	Ras-related rho (RhoA)	45.9
M93426	Protein tyrosine phosphatase zeta-polypeptide (PTPRZ)	8.0
AB001872	Leucine zipper–bearing kinase	4.0
AB001466	Efs1	2.9
Metabolic Enzymes		
U10473	p4betaGT/3 beta-1,4-galactosyltransferase	8.2
L31801	Monocarboxylate transporter 1 (SLC16A1)	5.1
U22961	Similarity to L-glycerol-3-phosphate-NAD oxidoreductase	3.9
AF040639	Aflatoxin B1-aldehyde reductase	3.9
Miscellaneous		
U16811	Bak	9.7
U52969	PEP19 (PCP4)	2.4
Nuclear		
U22376	Human (c-myb) gene	14.2
AF059194	MAFK	12.5
M21535	Erg protein (ets-related gene, HUMERG11)	5.7
L49219	RB1	5.5
X80878	R-kappaB	5.3

(CCL8) and ENA-78 (CXCL5) were upregulated as well.[35] Together, these chemokines bind to various G-protein–coupled chemokine receptors expressed by neutrophils, eosinophils, monocytes, and T cells and could attract a full range of leukocytes from the limbal region into the corneal stroma.[36]

Cellular Inflammation Genes

IL-1α regulation of genes expressed at the membrane fundamentally alters the fibroblast responsiveness to extracellular inflammatory factors. Membrane receptors for IL-7 and IL-15 were upregulated. Expression of gp130, a membrane receptor/signal transducer for IL-6,[37,38] increased fivefold, and together with an increase in IL-6, suggests the coordinated expression of a signaling pathway. Receptor responses to the cytokine glia-derived neurotrophic factor (GDNF) are modified by RETL-2, which increased threefold.[39] In contrast, the cytokine receptor for IL-11 was downregulated, suggesting a possible decreased responsiveness to the antiapoptotic signal generated by IL-11.[40] In addition, the decreased level of major histocompatibility complex (MHC) class II HLA-DR β-1 suggests that antigen presentation mediated by the fibroblasts may be diminished.[41]

Modulation of gene transcription is an essential feature of cytokine signaling, and expression changes in several transcriptional factors were observed (Tables 1, 2, 3). Of particular note was the increased expression of signal transducer and activator of transcription (STAT)-4 and R-κB. STAT proteins have dual functions: first binding to cytokine receptors and then migrating into the nucleus and directly activating transcription. STAT4 is essential for IL-12 signals that lead to production of interferon-γ.[42,43] The recently identified R-κB is a homologue of nuclear factor (NF)-κB, the prototypical inflammatory transcription factor. Although R-κB is known to regulate interleukin-2 receptor α-chain gene expression,[44] its conceivably broad transcriptional and inflammatory effects remain to be explored. In contrast to transcription factors, an alternative mechanism for gene regulation is the degradation of newly formed mRNA. The observed increase in the nuclear protein HEM45 may have this effect, as suggested by its putative role in degrading viral mRNA.[45]

An important mechanism of IL-1α–mediated inflammation is the activation of eicosanoid signaling by modulating arachidonic acid metabolism.[10] No effects were observed on phospholipase A2 or COX-2 expression. In contrast to the cyclooxygenase pathway, the lipoxygenase pathway appeared to be activated early with the observed increase in leukotriene C4 synthase.[46] Among other effects, its secreted product leukotriene-C4 (LTC4) increases microvascular permeability.[47]

Corneal Transparency and Extracellular Matrix Genes

One of the remarkable features of the cornea is its essential transparency to light—a transparency that is diminished after injury. This transparency depends on the extracellular collagen composition and organization, and corneal fibroblasts are a primary source of extracellular matrix proteins in the stroma. TGFβ, for example, regulates collagen synthesis and deposition and is an immunosuppressant in the anterior chamber.[48,49] Activation of TGFβ is dependent on thrombospondin-1, which increased fourfold after IL-1α treatment. This may affect both the matrix structure and regulation of inflammation.[50] An observed decrease in the TGFβ type III receptor suggests IL-1α may modulate specific TGF signaling pathways.[51,52] bone morphogenic protein (BMP)-7, which regulates specific collagen deposition, displayed a decreased expression.[53] In addition to these signaling molecules, IL-1α induced a threefold increase in

TABLE 3. Genes Undetectable after IL-1α Treatment

Accession Number	Gene Description	Fold-change
Extracellular		
Inflammation		
M28585	Leukocyte interferon alpha, clone pIFN105	−5.7
Matrix		
AJ224741	Matrilin-3	−3.1
X51801	OP-1 (BMP-7)	−3.1
Membrane Protein		
M29540	Carcinoembryonic antigen (CEA)	−6.3
M32578	MHC class II HLA-DR beta-1 mRNA (DR2.3)	−5.2
M69296	Estrogen receptor-related protein	−5.0
AF029343	Protocadherin 68 (PCH68)	−4.6
U49516	Human serotonin 5-HT2c receptor	−3.6
Cytosolic		
Structural		
U66582	GammaC-crystallin (CRYGC)	−13.2
AF052389	LIM domain binding protein (LDB1)	−12.5
D50370	Nucleosome assembly protein	−6.4
X95191	Delta-sarcoglycan	−6.1
S76756	4R-MAP2	−4.7
Signaling		
J03756	Growth hormone-variant-1 (GH1) and variant-2 (GH2)	−23.0
L11706	Hormone-sensitive lipase (LIPE) gene	−21.1
U07620	MAP kinase	−6.2
M60724	p70 Ribosomal S6 kinase alpha-I	−3.7
Miscellaneous		
AB015228	RALDH2-T	−12.5
D83017	Nel-related protein	−6.3
AJ005821	X-like 1	−5.1
Nuclear		
AF007833	Kruppel-related zinc finger protein hcKrox	−19.8
U66619	SWI/SNF complex 60 KDa subunit (BAF60c)	−15.7
U66561	Kruppel-related zinc finger protein (ZNF184)	−13.2
M93119	Zinc-finger DNA-binding motifs (IA-1)	−8.3
S82986	HOXC6	−6.9

α-1 type VII collagen (COL7A1), which has a specific role in anchoring the basal cells of the corneal epithelium to the stroma.[54] Also observed was a threefold decrease in α-5 collagen type IV (COL4A5), which helps form corneal basement membranes.[55]

Collagen matrix organization is regulated by accessory proteins. Proteoglycans maintain corneal transparency by regulating collagen fibril spacing and corneal hydration. A sevenfold increase in chondroitin 6-sulfotransferase–like protein was detected. Expression of this enzyme may be critical for maturation of the keratan sulfate proteoglycans, which are the major proteoglycans in the cornea.[56,57] At the same time, a fivefold decrease in the chondroitin sulfate proteoglycan versican was also detected. Other accessory proteins identified were matrilin-3, which forms collagen-dependent and -independent fibrils,[58] and thrombospondin-1.[59]

Secreted proteases are known to play a major role in remodeling the stromal extracellular matrix during corneal wound healing.[60–62] In response to IL-1α, fibroblasts displayed a 10-fold increase in human metalloelastase (HME/MMP-12), which was originally identified in macrophages.[63] MMP-12 has multiple extracellular matrix substrates and disrupts basement membranes.[64,65] No expression changes in membrane type matrix metalloproteinase-1, -2, or -4 were detected. Although MMP-1 gene regulation was not detected under these experimental conditions, the observed increase in manganese superoxide dismutase, and leukotriene C4 synthase may eventually signal an increase in MMP-1.[66,67] Secreted proteases are regulated in turn by a various inhibitors. RECK is a membrane protein that inhibits MMP-9 activity.[68] Decreased RECK suggests a concomitant increase in MMP-9 proteolytic activity, which is associated with corneal tissue destruction.[61,62] In contrast, IL-1α induced the increased expression of two protease inhibitors: squamous cell carcinoma antigen (SCCA) 2 (leupin) and PAI-2, which may help control the degradation of the stromal matrix.[69,70] There were no observed expression changes in tissue inhibitor of metalloproteinase-2, -3, or -4.

The avascular and relatively acellular quality of the stroma is essential to maintaining corneal transparency. Stromal neovascularization during inflammation and subsequent wound healing interferes with corneal light transmission, and IL-1 is implicated in this process.[71,72] Several angiogenic factors were identified after IL-1α treatment including endothelin (EDN)-1,[73,74] heparin-binding epidermal growth factor (HB-EGF),[75] GM-CSF-1,[76] and connective tissue growth factor (CTGF).[77] At the same time, changes in the potent angiogenic factor VEGF, which is not detected in stromal fibroblasts, was appropriately absent.[78,79] Similar to stromal neovascularization, proliferation of stromal fibroblasts interferes with corneal clarity. Some of the angiogenic factors, such as HB-EGF and CTGF, are also fibroblast mitogens.[80–82] Enhanced expression of the PDGF receptor may prime fibroblasts for PDGF mitogenic effects.[83] Increased expression of the follistatin-related protein (FLRG), a BMP-2 inhibitor, may protect specifically against BMP-2's proliferative effects.[84,85]

Cell Cytoskeletal and Motility Genes

Corneal fibroblasts normally lie flat between collagen lamella and extend filopodia to adjacent fibroblasts. After injury, however, filopodial extensions retract, actin networks form, and the cells become motile.[15,16] IL-1α is known to have a chemorepulsive effect on corneal fibroblasts in vitro and at the site of IL-1α injection in vivo.[83,86] The microarray identified several genes that may contribute to these processes.

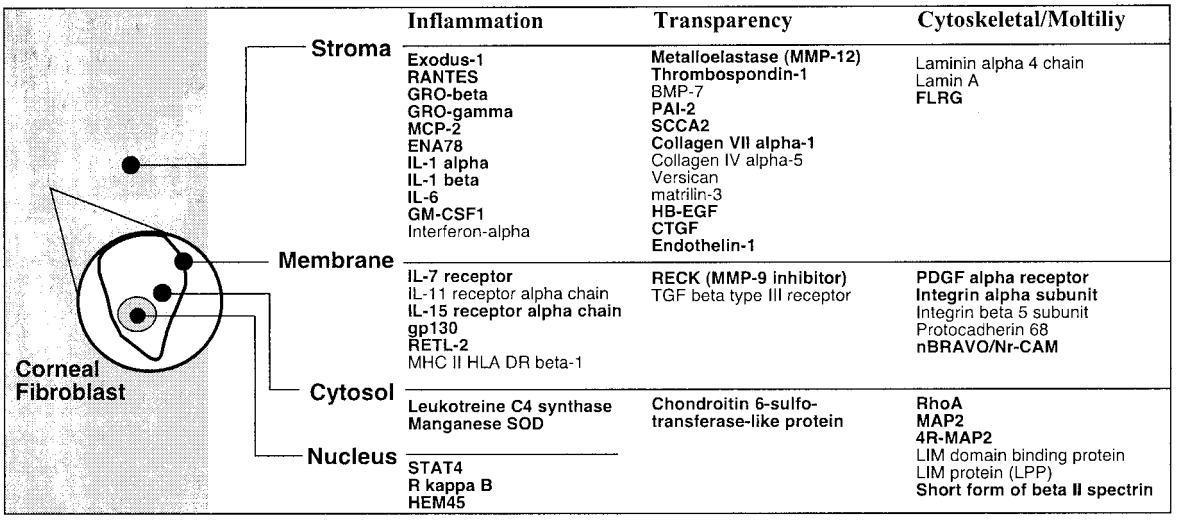

FIGURE 1. Model for IL-1α–mediated inflammatory response by corneal fibroblasts. IL-1α responsive genes with putative activities during inflammation, maintenance of corneal transparency, and modulation of the cytoskeleton and cellular motility are presented. Genes are arranged according to their predicted protein localization in various cellular compartments. Genes with increased expression (*bold type*) and decreased expression (*normal type*).

One of the most dramatic changes was the 46-fold increase in RhoA gene expression. The small guanosine triphosphatase (GTPase) RhoA is a signaling molecule that provides a link between extracellular signals and dynamic changes in the actin cytoskeleton.[87] It is associated with membrane retraction by the formation of actin stress fibers and focal adhesions and was recently identified in IL-1 receptor signal transduction.[88] Several intracellular proteins that directly regulate the cytoskeleton were recognized. These included actin-binding proteins, such as the LIM and the LIM domain binding protein (LDB)-1; the short form of β-II spectrin, α-catenin-like protein; and T-platstin.[89] Expression changes in microtubule-related proteins included alternatively spliced microtubule-associated protein (MAP)2 and the tubulin-binding protein RP-1.[90]

The transformation of quiescent keratocytes to mobile fibroblasts involves modulation of cellular interactions with extracellular matrix. Enzymes that degrade the matrix as previously described lead to loss of cell anchorage, whereas other matrix proteins, such as thrombospondin and PDGF signals, may promote motility.[83,91] BMP-2 and -4 are also chemoattractive signals, but no significant changes in gene expression were observed other than the increased BMP-2 inhibitor FLRG. Another component is the differential expression of cell adhesion molecules. A threefold decrease in HNK-1 sulfotransferase, which alters the cell adhesion activity of HNK-1, was detected along with a decreased expression of protocadherin 68. In contrast, increased expression of Nr-CAM/hBRAVO and syndecan-1 was observed.[92,93] Finally, transmembrane integrin proteins connect matrix molecules such as collagen and laminin to intracellular actin and transmit cell motility signals.[94,95] After IL-1α treatment, integrin-α expression increased, whereas integrin β-5 and laminin α-4 decreased. Together, these changes in gene expression may influence fibroblast cytoskeletal reorganization and motility during IL-1α mediated inflammation.

DISCUSSION

The pathophysiological mechanisms of corneal inflammation and how best to treat patients are poorly understood. Activation of the endogenous inflammatory response by IL-1 has both beneficial and detrimental effects.[5] After stromal injury, some degree of inflammation is essential for wound healing and control of infection. At the same time, however, inhibition of IL-1 may be favorable under certain conditions.[96,97] We hypothesized that identifying IL-1–responsive genes may help develop models for more specific therapeutic interventions. Studies with cultured cells are a well-established method for the initial identification of cytokine-responsive genes. Which corneal fibroblast phenotype represents the best therapeutic target remains to be determined,[8,98] and although the complex leukocyte effects are not represented, cytokine studies have nevertheless provided important insights.[6] The application of DNA microarray technologies, we expected, would identify additional cytokine responsive genes and help develop models for further investigation.[17,99]

A model for the response to IL-1α in corneal fibroblasts is presented in Figure 1. Genes thought to contribute to inflammation, maintenance of corneal transparency, and fibroblast motility and cytoskeletal rearrangement are emphasized and organized by their predicted protein localization. Such a model represents an exciting starting point for further investigation, yet it is critical to be aware of the limitations imposed by the model and inherent in microarray studies. The observed gene expression may reflect either increased transcription or increased mRNA stability, and whether the mRNA is translated into protein is not certain. Distinguishing tissue culture artifact from genuine expression changes requires in vivo studies. For example, Il-1α–responsive genes may be regulated by serum starvation or by an intrinsic IL-1α autocrine loop activated after trypsin-passage.[28] The expense associated with commercial DNA microarray analysis precludes the comprehensive study of time and dose responses, but the generation of custom gene chips containing only genes in the model will now allow such studies to be conducted at far less cost. Appropriate statistical tools have been lacking to interpret the seemingly thousands of changes in gene expression revealed by microarrays in the context of low replication, errors from multiple measurements, and determining significance by multiples of change. These pitfalls were avoided in this study with the use of the Cyber-T statistical software. Finally, it is important to recall that DNA microarray results do not reveal other important mechanisms that regulate proteins directly rather than through gene expression.

These caveats notwithstanding, DNA microanalysis was an effective and efficient screening method. Several previously known IL-1–responsive genes were identified, supporting the statistical and experimental methods. Some novel genes representing chemokines, chemotactic factors, cell adhesion molecules, interleukin receptors, matrix metalloproteinase, protease inhibitors, proteoglycans, angiogenic factors, cytoskeletal regulators, and transcription factors were identified. Each of these molecules represent potential targets, but what genes warrant further investigation? Regulation of MMPs is promising,[60] and this study correctly identified MMP-9 as an important candidate. Inhibition of MMP-9 has already shown some success as an adjunctive therapy in clinical studies,[100] and results suggest that targeting MMP-12, identified in this study, may also be important. Even with multiple redundant chemokine expression, targeting single chemokines or their receptors can dramatically reduce inflammation.[36] We identified chemokine targets not previously described in the cornea. Perhaps more important are the G-protein receptors (CXCR2 and CCR1, -2, -3, -5, and -6) implicated by this array of chemokines. It is interesting to speculate that therapies may be narrowed toward receptors expressed during leukocyte reactions specific to allergic, bacterial, or viral inflammation. Regulation of RhoA may control fibroblast motility and eventually wound contraction and corneal scarring, and specific inhibitors are available to test such a hypothesis. Finally, corneal gene therapy makes possible the targeting of specific transcription factors, such as STAT4 and R-κB, which have conceivably broad roles in inflammation.[101,102] Taken together, the observations based on this DNA microarray experiment provide fertile ground for developing and testing novel hypotheses to reduce cornea-related blindness.

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