Detection of Differentially Expressed Genes in Healing Mouse Corneas, Using cDNA Microarrays

Zhiyi Cao,[1] *Helen K. Wu,*[1] *Amenda Bruce,*[1] *Kurt Wollenberg,*[1] *and Noorjahan Panjwani*[1,2]

Purpose. To identify differentially expressed genes in healing mouse corneas by using cDNA microarrays.

Methods. Transepithelial excimer laser ablations were performed on mouse corneas, and the wounds were allowed to heal partially in vivo for 18 to 22 hours. Total RNA was isolated from both normal and healing corneas and was used for synthesis of cDNA probes. ^{33}P-labeled exponential cDNA probes were hybridized to mouse cDNA nylon arrays.

Results. Of the 1176 genes on the nylon arrays, the expression of 37 was upregulated and that of 27 was downregulated more than fivefold in the healing corneas compared with the normal, uninjured corneas. Interleukin (IL)-1β, laminin-5, and thrombospondin-1, which have been shown to be upregulated in healing corneas, were all found to be induced in the corneas in response to excimer laser treatment. Many genes were identified for the first time to be differentially regulated during corneal wound healing. Among the upregulated genes were intercellular adhesion molecule (ICAM)-1, macrophage inflammatory proteins, suppressors of cytokine signaling proteins (SOCS), IL-10 receptor, and galectin-7. Among the downregulated genes were connexin-31, a gap junction protein; ZO1 and occludin, tight junction proteins; and Smad2, a key component in the TGFβ signaling pathway. Microarray data were validated on a limited number of genes by semiquantitative RT-PCR and Western blot analyses.

Conclusions. Gene array technology was used to identify for the first time many genes that are differentially regulated during corneal wound healing. These differentially expressed genes have not previously been investigated in the context of wound healing and represent novel factors for further study of the mechanism of wound healing. (*Invest Ophthalmol Vis Sci.* 2002;43:2897–2904)

Durng the past decade, the potential of excimer laser keratectomy to modify corneal profile for correction of myopia has been realized.[1] Thousands of such procedures are performed each week, freeing individuals who have myopia from the need for eyeglasses and contact lenses. Although laser-assisted in situ keratomileusis (LASIK) is rapidly replacing photorefractive keratectomy (PRK) for correction of myopia, in certain cases, such as eyes with thin corneas and large pupils, PRK is still the procedure of choice. Also, because of potential concerns for corneal stability, with some cases of reported iatrogenic ectasia after LASIK,[2,3] PRK remains an attractive option in the refractive surgeon's armamentarium. In some cases, after PRK there is a delay in epithelial healing.[4,5] Such a delay is highly undesirable, because it puts the cornea at risk of development of postoperative haze, infectious keratitis, and ulceration. Also, delayed reepithelialization and failure of the migrated epithelium to remain adherent to the substratum after chemical injury or corneal infection are fundamental causes of debilitating clinical conditions, such as persistent or recurring epithelial defects and corneal ulceration.[6,7] Reepithelialization after injury and various surgical procedures takes place by the migration of adjacent cells over the injured area. The molecular events that mediate migration of epithelium over a wound and adhesion of epithelium to the underlying substratum have not been well defined. Another major wound healing–related complication is corneal scarring, which can occur not only after infection and trauma, but in some cases also after excimer laser surgery.[8,9] Corneal scarring can severely limit visual function and, at present, no effective pharmacologic agents are available to limit the development of scars. The molecular mechanism of scar formation also has not been fully elucidated. It is clear, however, that the processes of corneal epithelial and stromal wound healing are regulated by the coordinated expression of genes encoding growth factors,[10–12] cytokines,[13–15] extracellular matrix (ECM) molecules,[16–18] metalloproteinases,[19–24] specific glycoproteins,[25–27] and glycosyltransferases.[28] Investigators in past studies on the analysis of gene expression during corneal wound healing have used traditional approaches, including quantitative reverse transcription-polymerase chain reaction (RT-PCR), in situ hybridization, RNase protection assays, zymography, and Western blot and immunohistochemical analyses.[10–24,28] The disadvantage of these approaches has been that only a few genes are detected per assay. A more global approach is needed to elucidate the many factors that may play a role in the process of wound healing. In the present study, gene array technology was used to examine the differential gene expression in healing mouse corneas after transepithelial excimer laser keratectomy. A considerable number of genes were differentially regulated during reepithelialization of wounds, including several already published, confirming the potential value of gene array technology. We identified many genes for the first time to be differentially regulated during corneal wound healing. These differentially expressed genes have not been investigated in the context of wound healing and are novel factors for further study.

From the [1]New England Eye Center, Department of Ophthalmology and Center for Vision Research, and the [2]Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts.

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Corresponding author: Noorjahan Panjwani, Department of Ophthalmology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111; noorjahan.panjwani@tufts.edu.

Methods

Wound Healing Experiments

Throughout the study, 6- to 8-week old mice (C57BL/6 and 129 mixed genetic background) were used. All animal treatments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. To produce corneal wounds, we anesthetized mice by an intramuscular injection of 1.25% tribromoethanol (0.2 mL/10 g body weight). Tribromoethanol was

prepared by mixing 2.5 g 2,2,2 tribromoethanol and 5 mL 2-methyl-2-butanol (Aldrich Chemical Co., Milwaukee, WI) with 195 mL distilled water. Proparacaine eye drops (Alcain; Alcon Laboratories, Inc., Fort Worth, TX) were applied to the cornea as a topical anesthetic. Two-millimeter corneal wounds were produced on the right eye of each animal by transepithelial excimer laser ablations (2 mm optical zone; 42- to 44-μm ablation depth, PTK mode, Apex Plus excimer laser; Summit Technology, Waltham, MA). The left eye of each animal served as the control. After surgery, all animals received the pain killer buprenorphine intramuscularly (0.2 mL of 0.3 mg/mL; Buprenex; Reckitt and Colman Pharmaceuticals, Inc., Richmond, VA). Antibiotic ointment (Vetropolycin; Pharmaderm, Melville, NY) was applied, and the corneas were allowed to heal partially in vivo for 16 to 18 hours. At the end of the healing period, the wound areas were visualized by staining with methylene blue. The stained wound areas were then photographed and quantitated by computer (Sigma Scan software; SPSS Science, Chicago, IL).[29] For histologic analysis, eyes were enucleated, fixed in buffered formalin for 2 hours and were processed for preparation of paraffin-embedded sections and routine histology.

Gene Expression Analysis with cDNA Microarrays

Transepithelial excimer laser ablations (2-mm diameter) were produced on the right eye of 30 animals as described above. Corneas were allowed to heal partially in vivo for 18 to 22 hours. At the end of the healing period, animals were killed, and the corneas of both eyes were excised and immediately placed in liquid nitrogen and shipped to Clontech Laboratories, Inc. (Palo Alto, CA) for analysis of gene expression by cDNA microarrays. Because of the relatively low yield of RNA, especially from healing corneas, a PCR cDNA synthesis method (SMART PCR; Clontech Laboratories) was used for preparation of hybridization probes. This method is designed to enrich preferentially for full-length cDNAs with fairly accurate gene representation. Briefly, total RNA was isolated by using the reagents provided in a total RNA labeling kit (Atlas Pure Total RNA Labeling System; Clontech). For probe preparation, first-strand cDNA was synthesized with 175 ng RNA, a modified oligo(dT) primer (the CDS primer; Clontech), reverse transcriptase (Powerscript; Clontech), and oligonucleotides (SMART II; Clontech). Control probes involved incubation of samples without reverse transcriptase. The single-stranded cDNAs were exponentially amplified by long-distance (LD)-PCR. To determine the number of cycles needed to obtain a population of representative double-stranded cDNAs, aliquots of reaction products were collected at 15, 18, 21, and 24 cycles and were analyzed by agarose gel electrophoresis. Because cDNA synthesis was in the exponential phase throughout 15 to 24 cycles, 23 amplification cycles were deemed optimal for probe synthesis. The amplified exponential cDNAs (500 ng) were radiolabeled using Klenow enzyme and ^{33}P-α adenosine triphosphate (ATP), as described in the manufacturer's instruction manual (SMART cDNA Probe Synthesis for Atlas; Clontech).

The labeled probes were purified by filtration (NucleoSpin filter; Clontech) and were then hybridized to a mouse cDNA microarray (Atlas 1.2-1; Clontech). This is a broad-spectrum array, and it includes 1176 known mouse cDNAs, 9 housekeeping control cDNAs, and negative controls. After hybridization, the membranes were exposed to a phosphorescence imaging screen (Phosphorimager; Molecular Dynamics, Sunnyvale, CA). Hybridized dot intensities on the microarrays were quantified with image analysis software on computer (AtlasImage 2.0 software; Clontech). The first data analysis step was to determine the level of background activity. This was calculated as the median level measured in the blank spaces between panels of the array. Next, a threshold was calculated to differentiate positive, nonzero spot intensities due to gene expression from those due to nonbiological sources. This signal threshold is the minimum background-subtracted spot intensity that can be accepted as a true experimental result, rather than a signal due to experimental noise or other effects that are independent of the experimental treatment. The image analysis software sets this threshold as a percentage of the previously calculated background level. The default setting that was used in this analysis was twice the background (200%). To normalize the signal intensity between the two arrays, the global (sum) normalization method was used. The normalization coefficient was calculated with the following formula

$$\text{Normalization coefficient} = \frac{\sum_{N=1}^{n} (\text{intensity} - \text{background})_N}{\sum_{W=1}^{n} (\text{intensity} - \text{background})_W}$$

where N is the genes on array 1 hybridized with cDNA of normal corneas, W is the genes on array 2 hybridized with cDNA of healing corneas, and n is the number of genes on the array.

To normalize the data, intensity values of each spot on array 1 were divided by the normalization coefficient factor. The data on selected genes were verified by semiquantitative RT-PCR.

Semiquantitative RT-PCR

To confirm gene expression data obtained using cDNA microarrays, expression patterns of 24 selected genes was analyzed by semiquantitative RT-PCR performed with 0.5 μg total RNA preparations of the corneas and gene-specific custom primers purchased from Clontech and other reagents from a PCR kit (Advantage 2; Clontech). Annealing temperature was 68°C, and reactions were subjected to a varying number (n = 18–38) of cycles of PCR amplification. Amplified products collected at various cycles were analyzed by electrophoresis in 1.5% agarose-ethidium bromide gels. Intensity comparisons of the appropriate amplified products were made during the initial exponential phase of DNA synthesis.

Western Blot Analysis

Further studies were performed on one of the differentially expressed genes, *Smad2*. Western blot analysis was performed to determine whether Smad2 is differentially expressed in healing corneas at the protein level. For Western blot analysis, polyclonal rabbit anti-Smad2 (provided by Akiko Hata, Tufts University School of Medicine, Medford, MA) and monoclonal mouse anti-α-tubulin (Sigma, St. Louis, MO) were used. Protein extracts (20 μg) of normal and healing corneas of wild-type and galectin-3–deficient mice were prepared in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], containing 150 mM NaCl, 0.1% Nonidet P-40, and 0.5% deoxycholic acid) and were electrophoresed on 12.5% SDS-polyacrylamide gels. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) and were reacted with anti-Smad2 (1:1000 dilution) and the appropriate secondary antibodies.[28] Immune complexes were visualized with a chemiluminescence detection system (NEN, Boston, MA). The blots were subsequently stripped and reprobed with anti-α-tubulin antibody (diluted 1:100,000).

Results

Healing of Corneas

Excimer laser treatment removed epithelium and anterior corneal stroma (Fig. 1A). After injury, wound size progressively decreased with time (Fig. 1B). Complete reepithelialization of a 2-mm wound in vivo occurred between 24 and 28 hours. At the time of the harvesting of healing corneas for gene expression analyses, approximately 0.66- to 0.75-mm wounds remained de-epithelialized. To ensure minimal contamination with nonmigrating epithelium, care was taken not to let the corneas fully heal before collection of epithelium for RNA isolation.

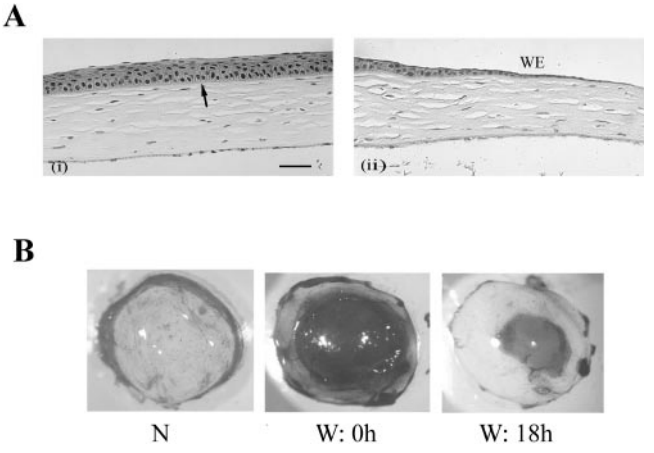

FIGURE 1. (**A**) Excimer laser injury removed epithelium and anterior corneal stroma. Two-millimeter corneal wounds were produced on the right eye of each animal by transepithelial excimer laser ablation (2-mm optical zone; 42- to 44-micron ablation depth, PTK mode). Immediately after injury, corneas were processed for histology and hematoxylin and eosin staining. (i) Control, uninjured cornea of the left eye. *Arrow* indicates epithelium. (ii) Excimer laser treated cornea of the right eye of the same animal. (**B**) Photographs of in vivo healing corneas used for gene expression analysis. The wound areas were visualized by staining with methylene blue immediately after wounding (W: 0h) or after healing in vivo for 18 hours (W: 18h). N, normal, uninjured cornea. *Dark* areas are cell-free wound areas; *light*, unstained areas are uninjured cellular areas. Wound size decreased progressively with time; complete reepithelialization occurred between 24 and 28 hours. For gene expression analysis (see Fig. 2 and Table 1), the animals were killed 18 to 20 hours after wounding and corneas excised and processed for RNA isolation. Bar, 50 μm.

Analysis of RNA and Exponential cDNA Probes

Yield of RNA from 30 normal and 30 healing corneas was 11.2 and 3.5 μg, respectively. The absorbance ratios (A_{260}:A_{280}) of the RNA preparations of the normal and healing corneas were 1.71 and 1.48, respectively. The ribosomal RNA 28S-to-18S ratio was 1.8 for both preparations. This ensured that the quality of the RNA preparation was satisfactory. The optimal number of PCR cycles required for the synthesis of the cDNA hybridization probes (SMART; Clontech) was determined to be 23. At the optimal number of PCR cycles, the cDNA remained in the exponential phase and the yield of the cDNA was 1 to 1.6 μg. As expected, amplified cDNAs appeared as weak smears on agarose gels (Fig. 2A, +RT). Within smears, a number of distinct bands, representing highly expressed genes, were seen. No amplified products were detected in control reaction mixtures incubated in the absence of reverse transcriptase (Fig. 2A, No RT).

Identification of Differentially Expressed Genes in Corneas after Excimer Laser Injury

To characterize genes that may be associated with the process of wound healing, we examined the expression levels of 1176 genes using broad-spectrum mouse cDNA expression arrays (Atlas; Clontech). In the phosphorescent images (Phosphorimager; Molecular Dynamics) of normal and/or healing corneas, 1110 genes exhibited a detectable hybridization signal, with 327 genes exhibiting only trace levels of signal (spot intensity: 5 units or less in both normal and healing corneas). To identify genes that are differentially expressed in the cornea in response to excimer laser treatment, we compared phosphorescent images of the arrays hybridized with ^{33}P-labeled cDNA probes generated from RNA preparations of normal and healing corneas (Fig. 2B) by using image-analysis software (AtlasImage ver. 2.0; Clontech). Spot intensities were normalized with the global normalization method with a normalization coefficient of 0.89. Initially, we analyzed expression levels of housekeeping genes. Normal-to-healing ratios of normalized spot intensities of six of the nine housekeeping genes present on the microarray was between 0.8 and 1.2 (ornithine decarboxylase, 1.0; β-actin, 0.8; ubiquitin, 0.8; 40S ribosomal protein, 0.9; 45-kDa Ca^{2+}-binding protein precursor, 1.2; hypoxanthine-guanine phosphoribosyl transferase, 0.8). Normal-to-healing ratios of the remaining three genes were slightly farther removed from 1.0, but in no case was the ratio greater than 2.0 (myosin 1α, 1.42; phospholipase A2, 0.62; GAPDH, 2.0). The global normalization method was therefore deemed satisfactory. Of the 1176 genes on the microarrays, the expression of 37 genes was upregulated and that of 27 genes was downregulated more than fivefold in the healing corneas compared with the normal, uninjured corneas. Genes showing greater than fivefold differential expression in healing corneas are listed in Table 1. For clarity, these genes are grouped according to their involvement in specific cellular tasks or properties. A list of all differentially expressed genes can be viewed on our Web site (http://www.neec.com/MicoarayData.html).

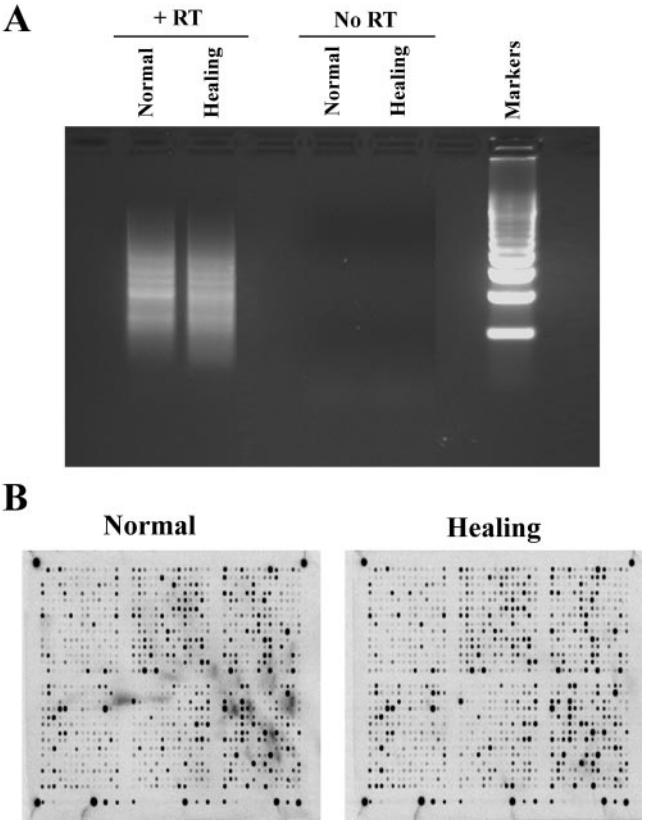

FIGURE 2. (**A**) cDNA probe synthesis. Total RNA (175 ng) isolated from normal and healing corneas was used for first-strand cDNA synthesis with reverse transcriptase (RT) and oligonucleotides. cDNA was amplified by LD-PCR with a predetermined exponential number of cycles. Aliquots of PCR products were electrophoresed on 1.2% agarose and ethidium bromide gel. +RT, RT-PCR performed with RT; No RT, control RT-PCR performed without RT. PCR products were detected only when RT was added to the reaction mixtures. Markers, 500-bp markers. (**B**) Phosphorescent images of cDNA mouse expression arrays. Arrays were hybridized with ^{33}P-labeled cDNA probes generated from total RNA of normal and healing corneas. Spot intensities were quantitated, and gene expression patterns of normal and healing corneas were compared using Atlas Image 2.0 Software.

TABLE 1. Genes Differentially Expressed during Corneal Epithelial Wound Healing

Multiple of Change	Gene Name	GenBank No.
Modulators of TGFβ function		
−15	**Smad2**	**U60530**
5	Thrombospondin-1 precursor (TSP1)	M87276
Growth factors/cytokines/chemokines and receptors		
170	**Interleukin-1 beta precursor (IL-1β)**	**M15131**
42	Interleukin-2 receptor gamma subunit (IL2RG)	L20048
12	Interleukin-1 receptor type II precursor (IL-1R; IL1RB)	X59769
100	Monotype chemoattractant protein-3	S71251
54	Macrophage inflammatory protein-1 beta (Act 2)	M35590
51	Macrophage inflammatory protein-1 alpha (MIP1α)	X12531
7	Platelet-derived growth factor receptor alpha precursor (PDGFR-α)	M84607
7	Macrohage inflammatory protein-2 alpha (MIP2-α)	X53798
−17	Coagulation factor II (thrombin) receptor (Cf2r)	L03529
−15	Wingless-related protein precursor (WNT4)	M89797
Cytokine suppressors		
66	**Suppressor of cytokine signaling protein-3 (SOCS3)**	**U88328**
27	**Suppressor of cytokine signaling protein-2 (SOCS2)**	**U88327**
10	**Interleukin-10 receptor precursor (IL-10R)**	**L12120**
12	Disabled homologue-2 (Drosophila)	U18869
Cell—cell adhesion and cell–matrix adhesion proteins/receptors		
−18	**Neural cadherin precursor (CDH2)**	**M31131**
−8	Gap junction beta-3 protein (GJB3); connexin 31 (CXN31)	X63099
−8	**Integrin beta-7**	**M95633**
−6	**Occludin (OCLN; OCL)**	**U49185**
36	Early T-lymphocyte activation 1 protein (ETA1)	J04806
32	**Intercellular adhesion molecule-1 precursor (ICAM1)**	**X52264**
19	LPS receptor (LPSR)	M34510
10	Collapsin-1 precursor; semaphoring D (SEMAD)	X85993
5	Integrin beta-2; CD18	X14951
5	**Galectin-7 (LGALS7)**	**AF038562**
13	Vascular cell adhesion protein-1	M84487
9	**Laminin gamma-2 precursor (LAMC2); kalinin/epiligrin**	**U43327**
Transcription activators and repressors		
14	Enhancer of zeste homologue-2 (EZH2)	U52951
9	**Transcription factor reIB**	**M83380**
7	Myb-related protein B (B-myb)	X70472
−206	GA-binding protein beta-2 subunit (GABP2)	M74517
−5	Transcription initiation factor (TFIID)	D001034
−6	Brahma-related protein-1 (BRG1)	S68108
−6	Chromobox homologue-4 (CBX4)	U63387
−5	Ear-2; v-erbA related proto-oncogene	X76654
Neuropeptides and neurotransmitter receptors		
13	5-hydroxytryptamine (serotonin) receptor 2c	Z15119
−34	Proenkephalin B precursor	AF026537
−10	Proenkephalin A precursor	M55181
Cytoskeletal proteins		
7	Beaded filament structural protein in lens 1 (BFSP1)	Y13602
11	Keratin 18 (KRT18); keratin D (KERD)	M11686
Proteases and protease inhibitors		
5	Serine protease inhibitors 2-2 (SPI2-2)	M64086
5	Matrix metalloproteinase-2 (MMP2)	M84324
−13	Plasma kallikrein precursor (serine protease)	M58588
Tumor suppressors		
−52	**PMS2 DNA mismatch repair protein**	**U28724**
−17	**RAB19, member RAS oncogene family**	**X80473**
−7	**Tight junction protein ZO-1; (TJP1)**	**D14340**
−6	Ezrin; villin 2	X60671
Symporters, antiporters and xenobiotic transporters		
−11	5HT transporter (5HTT)	AF013604
−10	GABA-A transporter-3	L04663
−6	Excitatory amino acid transporter-3 (EEAC1)	U73521
−31	Plasma glutathione peroxidase precursor (GPX3)	U13705
−5	Glutathione *S*-transferase A	D01034
Cell-cycle–regulating kinases and other proteins		
5	Cyclin-dependent kinase-5 (Cdk5)	D29678
6	Calcyclin-binding protein	U97327
Miscellaneous transducers, effectors, and modulators		
11	Glutamate receptor channel subunit gamma	X04648
6	Hemopoietic cell kinase (HCK)	Y00487
9	Osmotic stress protein Osp94	D49482
73	Histidine decarboxylase (HDC)	/X57437
12	TRAF-associated NF-κB activator (TANK)	U51907
10	Src-like adapter protein; (SLAP)	U29056
−7	Transducin beta-2 subunit	U34960
−14	Inositol 1,4,5-trisphosphate receptor type 2	Z71173
−9	Protein tyrosine phosphatase	D83966

Results of genes marked in bold were confirmed by semiquantitative RT-PCR. Normal: healing ratios for housekeeping genes were: 1.0 (ornithine decarboxylase); 0.9 (40S ribosomal protein); 0.8 (β-actin); 0.8 (ubiquitin); 0.8 (hypoxanthine-guanine phosphoribosyl transferase); 1.2 (45-kDa Ca^{++}-binding protein precursor); 0.62 (phospholipase A2); 1.42 (myosin); 2.0 (GAPDH).

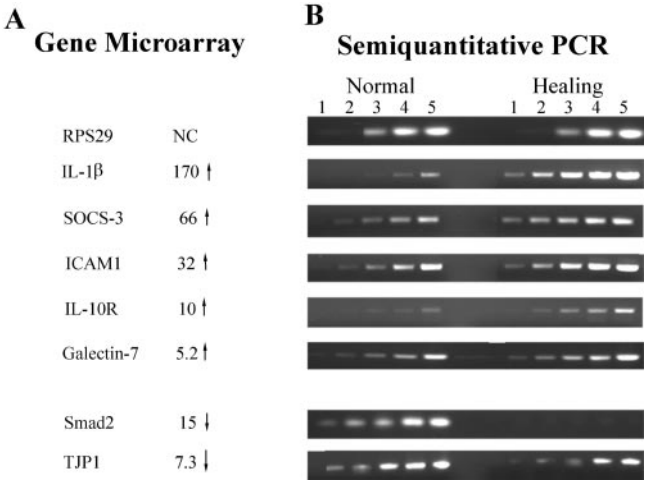

FIGURE 3. Comparison of differential gene expression data obtained by array hybridization and semiquantitative RT-PCR. (**A**) Tabulated results of microarray data of selected genes. *RPS29* is a housekeeping gene. *Arrows*: fold up or down, in healing corneas compared with normal corneas. RPS29, ribosomal protein S29; IL-1β, interleukin-1β precursor; SOCS-3, suppressor of cytokine signaling protein-3; ICAM-1, intercellular adhesion molecule-1 precursor; IL-10R, interleukin-10 receptor precursor; Smad2, mad related protein-2; TJP1, tight junction protein-1 (ZO1). NC, no change. (**B**) Confirmation of microarray data by semiquantitative RT-PCR. *Lanes 1–5*: amplified products collected at 18, 23, 28, 33, and 38 cycles, respectively, for *RPS29* (Normal) and 30, 32, 34, 36, and 38 cycles, respectively, for other genes (Healing). Note that semiquantitative RT-PCR data are consistent with array hybridization data.

Among approaches used to validate the microarray data, use of duplicate arrays, quantitative or semiquantitative RT-PCR, and Northern blot analysis may be included. In view of the fact that errors have been found in 25% to 60% of the probes on the Affymetrix U74 mouse chip (Affymetrix, Santa Clara, CA),[30] it appears that even if duplicate or triplicate arrays are used in analyses, it is essential to validate the data by techniques other than the use of microarrays. Published literature validating microarray data by alternative approaches has been sparse. We used gene-specific semiquantitative RT-PCR to confirm the differential expression of 21 selected genes. We chose these genes only because they are differentially expressed and can be taken as examples to confirm the reliability of Atlas (Clontech) hybridization. Three housekeeping genes were used as controls. Representative examples of the RT-PCR results are shown in Figure 3. The semiquantitative RT-PCR showed expression patterns very similar to those obtained by Atlas array hybridization for 18 of the 24 genes tested (~75%). Semiquantitative RT-PCR experiments were performed at least three times for each of the 24 selected genes, with reproducible results. Again, no PCR products were amplified in control reactions carried out in the absence of RT with oligonucleotides (SMART II; Clontech), CDS primer, and LD-PCR (Fig. 2).

Initially, we analyzed expression patterns of genes that are known to be differentially expressed in the cornea in response to injury. Interleukin (IL)-1β,[14] laminin-5,[19,25] and thrombospondin-1,[26] which have been shown to be upregulated in healing corneas, were all found to be induced in the corneas in response to excimer laser treatment (Table 1), thereby further attesting to the validity of the analysis. Next, we analyzed expression patterns of genes for which regulated expression in response to injury has not been studied in the cornea, but has been well characterized in nonocular tissues such as skin and brain. Expression of IL-2 receptor (IL-2R),[31] intercellular adhesion molecule (ICAM)-1,[31] and macrophage inflammatory proteins (MIP) I and II,[32] which are known to be induced in response to either epidermal and/or spinal cord injury, was induced in response to excimer laser injury of the corneas (Table 1). Similarly, a gap junction protein connexin-31,[33] and a tight junction protein ZO1,[34] which are known to be repressed during epidermal and corneal endothelial injury, respectively, were both downregulated in the corneas after excimer laser treatment.

We then searched for differentially expressed genes that have not been previously reported and/or investigated in the context of wound healing. We identified many genes for the first time to be differentially regulated during corneal wound healing. Those genes included a variety of cytokine suppressors, transcription factors, cell adhesion molecules, cytokines, neuropeptides, tumor suppressors, and cell cycle regulating kinases (Table 1). We emphasize, however, that before undertaking future studies on any gene identified by the gene array method, results should be confirmed by more traditional means, such as quantitative RT-PCR or Northern blot analysis. Also, the extent of change in the expression level of mRNA transcripts and the respective proteins may not be identical for a variety of reasons, such as variation in the RNA and protein turnover rates and stability. Therefore, results should be further verified at the protein level by Western blot analysis or other appropriate techniques.

We performed further studies at the protein level on Smad2, a key component in the TGFβ signal-transduction pathway, which was found by cDNA microarray to be downregulated during corneal wound healing (Table 1) and semiquantitative RT-PCR (Fig. 3) analyses. For this, detergent extracts prepared from 14 normal and 14 healing corneas were analyzed by Western blot analysis. These analyses revealed that healing mouse corneas also expressed reduced amounts of Smad2 protein compared with normal corneas (Fig. 4). The Western blot analysis was performed at least twice on each sample with reproducible results.

DISCUSSION

In the present study, gene array technology was used to examine differential gene expression in healing mouse corneas. For this study, a relatively low-density nylon filter microarray containing approximately 1200 genes was used. Despite the modest number of genes contained on these microarrays, we were able to verify previously known differentially expressed genes as well as to identify previously unknown differentially ex-

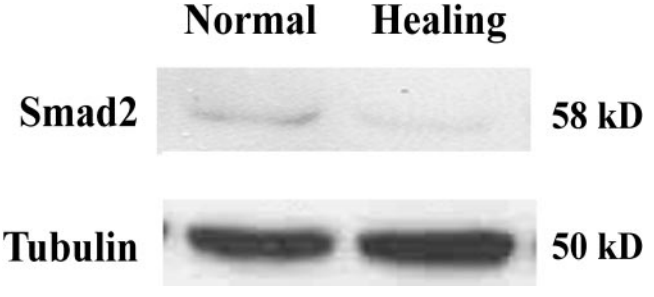

FIGURE 4. Western blot analysis showing that healing corneas express reduced level of Smad2 protein. RIPA buffer extracts of normal and healing corneas representing 20 μg protein were loaded in each lane and electrophoresed on SDS-polyacrylamide gels. After immunostaining with antibody to Smad2, the blot was striped and subsequently reprobed with antibody to tubulin. Western blot analysis was performed at least twice on each sample with reproducible results. The healing corneas contained a reduced level of Smad2 (58 kDa) compared with normal corneas.

pressed genes in healing corneas. Although it is beyond the scope of this report to highlight the potential significance of each altered gene, some are of unique interest and warrant specific mention. As has been shown by others, we found that injury triggers the induction of proinflammatory cytokines. Cytokines secreted in response to injury regulate important cellular processes but, if left uncontrolled, they can cause extensive damage to the tissue. Although key events in cytokine signaling are well defined, less is known about how the cytokine signal transduction and synthesis are switched off. To this end, in the present study, we found that in response to corneal injury, expression of suppressors of cytokine signaling (SOCS)-2, SOCS-3, and IL-10R was induced. SOCS proteins act as negative regulators of a key cytokine-activated signaling pathway, the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, which is used by many cytokines, including IL-6, IL-2, and IFNγ.[36–38]

IL-10 has been identified as a cytokine synthesis inhibitory factor (CSIF) and is also known as macrophage deactivating factor.[39] IL-10$^{-/-}$ mice exhibit severe chronic inflammatory bowel disease and other exaggerated inflammatory responses. IL-10 strongly inhibits the production of IL-1α, -1β, -6, -8, -10 itself, and -12; granulocyte-macrophage colony stimulating factor (GM-CSF); TNFα; MIP-1; and -2; regulated on activation normal T-cell expressed and secreted (RANTES); and leukemia inhibitory factor (LIF)[39] and has been shown to suppress proinflammatory mediators and inflammation in the herpes simplex factor (HSV)-1 infected cornea.[40,41] Based on reports showing that the expression level of IL-10 does not change significantly after corneal injury[15] and our finding that IL-10R precursor levels are increased after corneal injury, we speculate that the expression level of IL-10R rather than IL-10 itself may control the extent of inflammatory response after corneal injury.

It is well established that in response to injury to the epithelium, cells at the leading edge undergo a phenotypic conversion characterized by a dramatic reorganization of the cytoskeleton. In the present study, among the group of cytoskeletal proteins, the expression of genes encoding beaded filament structural protein (BFSP) and keratin 18 (K18) was induced after excimer laser keratectomy. It is known that injury to epidermis and other stratified epithelia triggers induction of a number of keratins including K6, K16, and K17. It is thought that in the context of epidermal wound healing the function of these keratins may be to promote reorganization of the cytoplasmic array of keratin filaments, an event that precedes the onset of keratinocyte migration into the wound site.[42–44] Whether K18 promotes a similar function preceding the onset of reepithelialization of corneal wounds remains to be determined. Reorganization of the cytoskeleton after injury is associated with disruption of stable intercellular adhesion and redistribution of adhesion-related molecules. In fact, the breakage of the stable intercellular contacts is prerequisite for initiating the phase of reepithelialization. Molecular mechanisms responsible for the dissolution of intercellular contacts have not been fully elucidated, but it is known that expression levels of a gap junction protein connexin-31 and a tight junction protein ZO1 are downregulated during epidermal[33] and corneal endothelial[34] wound healing, respectively. In the present study, we found that in addition to connexin-31 and ZO1, another tight junction protein, occludin, is also downregulated during corneal wound healing after excimer laser treatment.

Cell–matrix interactions clearly play a key role in reepithelialization of wounds. In this respect, in the present study, we found that the expression of genes encoding laminin-5 and galectin-7 was induced in the corneas after excimer laser injury. Both laminin-5 and galectin-7 have potential to play a key role in mediating cell–matrix interactions and cell migration. In corneal[19,25] as well as in cutaneous[45] models of wound healing, precursor laminin-5 is deposited under migrating epithelium into a basement membrane–like structure at very early stages after wounding, and mAbs to endogenous (unprocessed) laminin-5 have been shown to inhibit human corneal epithelial cell migration.[46] As regards galectin-7, it is a member of the galectin class of β-galactose-binding proteins. Galectins are found on the cell surface and within ECM and are thought to influence cell–matrix adhesion by binding to the ECM and cell surface glycosylated counter receptors (e.g., certain isoforms of laminin, fibronectin, vitronectin, and integrins).[47–50] Galectin-7,[51,52] first reported in 1994,[49] is not as well characterized as some other members of the galectin family, such as galectin-1 and -3. Several studies have implicated galectin-7 in the apoptotic process,[53,54] and recent studies in our laboratory have suggested that galectin-7 most likely also plays an important role in reepithelialization of wounds. We found that, similar to mRNA transcripts, protein levels of galectin-7 are also elevated in corneal epithelium after injury and that exogenous galectin-7 stimulates reepithelialization of corneal wounds in organ culture (Panjwani et al., manuscript submitted).

In the present study, we demonstrated for the first time that Smad2 is markedly downregulated in the corneas in response to excimer laser injury. Smad2 and its closely related homologue, Smad3, play a pivotal role in the intracellular function of TGFβs. Studies by Ashcroft et al.[55] and Koch et al.[56] have demonstrated that the rate of reepithelialization of cutaneous wounds is accelerated in Smad3-null mice as well as in TGFβ1-null mice. These studies suggest that the downregulation of the function of TGFβ accelerates the process of reepithelialization of wounds. This notion is consistent with the findings reported in the present communication that after excimer laser injury, reepithelialization of wounds is associated with downregulation of Smad2. However, we note that Smad2 and Smad3 have distinct downstream signal-transduction pathways[57] and distinct phenotypes (e.g., Smad2, but not Smad3, is embryonic lethal), and in the study by Ashcroft et al.,[55] Smad2 heterozygotes did not show acceleration of reepithelialization of cutaneous wounds. This may suggest that in the case of Smad2, downregulation by twofold, as would be expected in heterozygotes$^{+/-}$, may not be sufficient to accelerate reepithelialization of wounds. In the present study, by microarray analysis, Smad2 mRNA transcripts were downregulated by 15-fold in response to corneal injury.

The unanticipated observation of the present study is that the gene expression pattern of most of the growth factors and growth factor receptors was not significantly different between normal and healing corneas. Although this may appear to be in conflict with a report by Wilson et al.[10] showing that expression of keratinocyte and hepatocyte growth factors is induced in response to scrape injury in the mouse cornea, it is likely that the gene expression pattern in healing corneas varies, depending on the nature of the injury (e.g., scrape injury[10] vs. excimer laser keratectomy). Differential expression of other growth factors may have escaped detection for a variety of reasons. One possibility is that, in our study, the elevation of growth factors and/or their receptors occurred and returned to normal levels before the corneas were harvested (18–22 hours). For example, Zieske et al.[58] have shown that expression of amphiregulin, an epidermal growth factor (EGF) receptor ligand, is induced in rat corneas at 4 and 8 hours after injury, but not thereafter. Equally likely is the probability that expression levels of some growth factors such as TGFβ may increase several days or even weeks after injury. In this respect, Kaji et al.[12] have shown in a feline model, that expression levels of TFGβ1, -β2, and -β3 and TFGβ receptors were elevated at 4 weeks, but not at 1 week, after PRK.

Our findings that neuropeptide precursors proenkephalins A and B, and a number of symporters and antiporters including 5HT transporter, γ-aminobutyric acid (GABA)-A transporter-3, and excitatory amino acid transporter-3, are all expressed in reduced amounts in healing corneas most probably suggest significant damage to neural processes of the cornea in response to excimer laser treatment. In this respect, it is well established that patients experience dry eye symptoms for 6 to 12 months after PRK.[59,60] This is thought to be due to damage to the nerve plexus in the corneas. Also, confocal microscopy has shown that after PRK there is significant nerve damage with regeneration of nerves within 6 to 12 months.

In summary, in the present study, with the use of gene array technology, we demonstrated that wound healing response after excimer laser injury is characterized by production of proinflammatory cytokines in concert with induction of cytokine suppressors and cell–matrix adhesion molecules, while simultaneously downregulating production of cell–cell adhesion molecules and Smad2, a key component of the TGFβ signal-transduction pathway. Many differentially expressed genes identified in this study have not been previously investigated in the context of wound healing and represent novel factors for further study of the mechanisms of wound healing. Although the transcriptional profile presented herein represents more than 1000 genes, our analysis of differential gene expression during corneal wound healing is far from complete. The total number of genes analyzed in this study represents less than 5% of expressed mouse sequences. Moreover, our results reflect only changes in steady state mRNA levels. Post-translational modification events that may also play a key role in the wound-healing process remain to be investigated.

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