A Time Course Microarray Study of Gene Expression in the Mouse Lacrimal Gland after Acute Corneal Trauma

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PURPOSE. To investigate the effect of corneal trauma on gene expression in the lacrimal gland and to assess how many genes and what specific genes are regulated in response to corneal trauma.

METHODS. A mouse model with acute corneal trauma was created with a chemical burn to the cornea with silver nitrate. Sixty-four female BALB/c mice at 12 weeks of age were randomly divided into eight groups, eight mice per group. The corneas of four mice in each group were bilaterally cauterized with silver nitrate, and another four time-matched mice were used as the control. The total RNA of the lacrimal gland was then extracted, at eight time points— 0.5, 1, 3, 8, 24, 72, 120, and 360 hours—after the corneal burn, and gene expression was examined with using cDNA microarray technology.

RESULTS. Evaluation of 15,065 genes with multiple array replications showed significantly altered expression in 3,799 genes at one or more of the eight time points. Of those, 1,528 were known genes and 2,271 were unknown. The analysis of known genes showed broad and long-lasting gene suppression in most functional gene groups, including housekeeping, energy metabolism, protein degradation, DNA and protein synthesis, and apoptosis-associated genes. Heat shock genes were upregulated beginning at the 8-hour time point, indicating a stress response.

CONCLUSIONS. This study demonstrates that corneal trauma has profound effects on the regulation of gene expression in the lacrimal gland and may provide genetic evidence for a cornea-to-lacrimal gland feedback mechanism in dry eye. (*Invest Ophthalmol Vis Sci.* 2005;46:461–469) DOI:10.1167/iovs.04-0677

A highly integrated organism, such as a mammal, has very sophisticated control systems to regulate and integrate its functions as a whole, to adapt to environmental changes, to operate autonomic regulatory mechanisms, and to maintain homeostasis. The relationship between organs is coordinated through the nervous system and/or endocrine system in a spatial and temporal manner by such mechanisms as the neural reflex arc and the neuroendocrine response.[1] The interaction between the cornea and the lacrimal gland is one example of the way the neural reflex is responsible for coordinating the

functions of both organs. Under normal physiological conditions, sensory nerves in the cornea transmit an afferent stimulation signal to the brain stem and then, after a series of interneurons, the efferent signal is transmitted to the lacrimal gland through the parasympathetic and sympathetic nerves that innervate the gland and drive tear production and secretion.[2,3] A normal tear flow and tear film is needed to maintain the health of the cornea and the ocular surface.[4–6] Thus, the neural circuit constitutes a functional unit of the cornea and lacrimal gland. Damage to this circuit interrupts the normal neural regulation of lacrimal gland secretion and causes dry eye syndromes, one of the most common and significant problems in the eye clinic.[3,4] Evidence for this mechanism comes from the clinical observation that dry eye syndrome frequently occurs after corneal trauma, such as in corneal refractive surgery or contact lens wearing.[7–10] Damage to cornea sensory nerve terminals that creates partial denervation is hypothesized to be the main cause of dry eye syndrome after refractive surgery and normal aging.[3] Clinical studies confirmed that tear production and secretion are reduced after in situ keratomileusis (LASIK) corneal surgery, indicating dysfunction of the lacrimal gland.[8,10,11] Corneal injury, or overstimulation, is known to alter gene expression in the lacrimal gland. Earlier experiments identified several growth factors and other genes that were differentially expressed in the lacrimal gland after creation of a corneal epithelial defect.[12,13] Tumor necrosis factor (TNF)-α mRNA in the rabbit lacrimal gland was upregulated by corneal wounding.[14]

Therefore, it appears that regulation of lacrimal gland gene expression in response to the corneal injury is involved in the creation of dry eye syndrome through partial denervation, overstimulation, or both. However, we have known little about how many genes and what specific genes in the lacrimal gland are altered by corneal injury besides the genes just listed. Multiple gene families may be involved in creating the defective lacrimal gland function that leads to dry eye after corneal trauma. In the present study, using a mouse model of corneal trauma and large-scale cDNA microarray technology,[15–17] we investigated the gene expression profile of the lacrimal gland after an acute chemical burn injury to the cornea. The chemical burn not only damages the cornea and its nerves, but also creates an indirect, yet predictable overstimulation of the lacrimal gland through the neuronal circuit between the two organs. This study has the potential to present a new picture of the gene expression profile in the lacrimal gland in response to corneal trauma by examining a wide variety of genes simultaneously in large scale. This approach will provide an opportunity to explore specific candidate genes in parallel in connection with corneal injury. The time course observations allow us to elucidate the whole process of such a response during a specific period.

MATERIALS AND METHODS

Animal Model

Female BALBc mice (The Jackson Laboratory, Bar Harbor, ME), 12 weeks of age, were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the exper-

FIGURE 1. Microphotographs of control (**A**) and silver nitrate–burned (**B**) mouse corneas. *Dotted line:* outline of burned area on the cornea, approximately 2 mm in diameter. Scale bar, 1 mm.

imental protocol used in this study was approved by Institutional Animal Care and Use Committee (IACUC) of Oregon Health and Science University (OHSU). A mouse model of acute corneal trauma was created by inducing chemical burns to the cornea of both eyes of the experimental animal with silver nitrate, in accordance with previous reports,[18,19] with modifications. The procedure immediately caused damage to the cornea as showed in Figure 1. The silver nitrate burn has profound effects on the cornea, inducing edema, neovascularization, and opacification.[18] Histologically, the corneal thickness doubles due to edema and the corneal endothelial and epithelial cells are lost at the site of the corneal edema after a silver nitrate burn.[18] The procedure was as follows: Mice were put under anesthesia (2% isoflurane in oxygen; Abbott Laboratories, North Chicago, IL), and their eyes were observed under a microscope for treatment with silver nitrate applicator activated with 3 μL of sterile water (37.5 mg silver nitrate and 12.5 mg potassium nitrate; Graham-Field, Inc., Bay Shore, NY). Our standard procedure was to perform two burns per eye, each with a separate applicator: one burn on the limbus, including some conjunctiva and peripheral cornea, for 1 second and the other burn on the cornea slightly off center and away from the limbal burn for exactly 5 seconds. Both burns were followed by an immediate rinse with sterile saline. For animals observed for >24 hours, eyes were then treated with gentamicin ointment (Schering-Plough Corp., Kenilworth, NJ) to minimize infection. For each experimental animal, a matching control mouse had a sham burn (handled the same except for the burn). The animals were killed at eight time points—0.5, 1, 3, 8, 24, 72, 120 and 360 hours—after corneal trauma, and the lacrimal glands were dissected. The investigation at each time point included four treated and four control animals.

RNA Extraction

The total RNA was extracted from fresh lacrimal gland pretreated with preservative (RNAlater) in an RNA extraction kit (RNAqueous; Ambion Inc., Austin, TX). Tissue from both control and treated animals was processed together by identical procedures. The tissue was homogenized with a mechanical homogenizer (Polytron PT3000; Technical Laboratory Services, Toronto, Ontario, Canada) in lysis solution, and the RNA was extracted and purified through a spin column included in the kit. The extracted RNA was treated with DNase to eliminate DNA contamination, quantified by UV spectrophotometer, and subjected to 1% denatured agarose gel electrophoresis in a MOPS (3-(*N*-morpholino)-propanesulfonic acid) buffer (Ambion Inc.) containing 6.66% formaldehyde (Sigma-Aldrich, St. Louis, MO), to assess the integrity of the RNA. The RNAs from four animals at each time point were pooled to reduce the effect of biological variation and to improve efficiency according to previous investigations.[20,21]

Microarray Preparation

Microarrays were printed and hybridized in the Spotted Microarray Core (SMC) of the OHSU Gene Microarray Shared Resource. Complete protocols and array details for microarray chips are available from the SMC Web site at http://www.ohsu.edu/gmsr/smc. The source library

for the mouse arrays was the National Institute on Aging (NIA) 15K library,[16] which can also be accessed at the NIA 15K Mouse cDNA Clone Set Web site of the NIA/NIH Mouse Genomics Home Page at http://lgsun.grc.nia.nih.gov/cDNA/15k.html. Briefly, individual clones were amplified (DyNAzyme EXT; MJ Research, South San Francisco, CA) in thermocyclers (DNA Engine Tetrad; MJ Research). PCR products (average size, 1.5 kb) were separated from unincorporated material, dried, and then resuspended in 15 μL of Tris/EDTA (TE) buffer. Fifteen microliters of dimethyl sulfoxide (DMSO) were added to each PCR product, which was then stored at $-20°$C until use. Printing plates containing 3 μL of PCR product in TE/DMSO per well were prepared and used for up to seven printings (Cartesian PixSys 5500XL array printer; Genomic Solutions, Ann Arbor, MI), by using CMP-3 array pins, with 16 pins corresponding to 16 grids per array (TeleChem, Sunnyvale, CA) onto microarray slides (UltraGAPS; Corning-Costar, Corning, NY). Printed arrays were baked at 80°C for 3.5 hours, and then stored desiccated in the dark until use. Genes are printed in duplicate on each slide. The mouse library is represented on two separate microarray chips: chip A with 8271 genes, and chip B with 6794 genes. In addition, each chip has multiple copies of each of 10 plant genes (Stratagene, La Jolla, CA) and buffer-only controls. Random samples from each print batch were examined using fluorescent staining (Syto61; Molecular Probes, Eugene, OR) for quality control.[22]

Target Labeling and Array Hybridization

The target was generated from total RNA by using a microarray labeling system (Tyramine Signal Amplification [TSA]; PerkinElmer, Boston, MA) described previously.[17] The complete protocol, including modifications unique to the OHSU SMC, is available at the SMC Web site at http://www.ohsu.edu/gmsr/smc/smc_downloads.shtml. TSA is an indirect labeling protocol that avoids the problem of incorporation bias. Other items, such as the robustness of fluorescence, are controlled at later stages by adjustments in laser power, by photomultiplier tube (PMT) gain, and by intensity-based normalization (described in subsequent sections). SMC data have been verified by Northern blot analysis (Reddy PH, personal communication, 2003). Briefly, either fluorescein- or biotin-modified dCTP was incorporated into cDNA generated from a total RNA template. The RNA was degraded and the cDNA hybridized to the arrays in 60 μL of hybridization buffer under coverslips (Lifter-Slip; Erie Scientific, Portsmouth, NH). Array chips were blocked with 2% BSA in 2$\times$ SSC at 65°C for 1 hour before use, and hybridization was initiated within 30 minutes of blocking. Each pair of samples was hybridized to the array chips at 65°C overnight. Five or six identical replicates of the array chips were hybridized for every time point in this study. Hybridized material was detected by using an SMC-developed modification of the TSA detection protocol. After a blocking step, array chips were incubated with horseradish peroxidase (HRP)–linked streptavidin and then incubated with Cy5-tyramine for signal development. The arrays were treated to inactivate the HRP and then incubated with HRP-linked anti-fluorescein antibody followed by Cy3-tyramine. The arrays were never allowed to dry between incubations. This reduced background to approximately 250 U on a scale from 0 to 64,000, with little variation in background across the array.

Imaging and Image Analysis

Arrays were quickly scanned once at low resolution (ScanArray 4000XL; PerkinElmer) with accompanying software (ScanArray Express; PerkinElmer) and the laser intensity was adjusted to bring the brightest spot below saturation when the data were analyzed, with 5% trimming of the highest and lowest pixels in the spot area. Gain was maintained at a constant 80% maximum. The Cy5 channel was scanned before the Cy3 channel. Gridding and quantitation were performed on computer (ImaGene ver. 4.x or 5.0; BioDiscovery, El Segundo, CA). Each gridding was visually examined and anomalous spots were flagged for exclusion. Occasional runs resulted in quenching of signals for a few spots at high intensity. These spots were obvious in scatter-
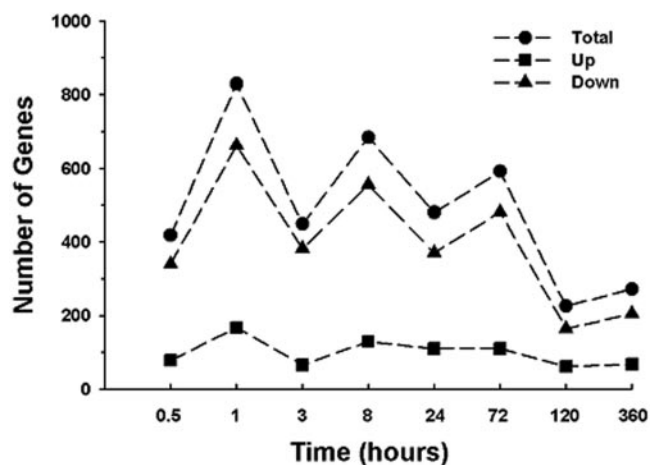
FIGURE 2. Time course of the number of known genes with significantly altered expression and >1.5-fold change. The curves depict the total number of genes with altered expression (upregulation and downregulation) at every time point. The greatest number of genes was altered at 1 hour. Downregulation was predominant over upregulation at all time points.

grams of $\log_2$ (Cy5) versus $\log_2$ (Cy3) and in M versus A plots and were flagged for removal from the data sets.

Preprocessing and Statistical Analysis of Microarray Data

Data preprocessing and statistical analysis were performed using an in-house routine written in R statistical language.[23] The mean of the signal intensities in each channel of a spot was subtracted from the median of corresponding local background intensities. The local background-corrected signal intensities were transformed into base-2 logarithms. An intensity-dependent scale normalization by 16-pin groups within an array chip, similar to the methods proposed by Yang et al.,[24] was applied to the log-transformed, local background–corrected intensities, as an intensity-dependent normalization can remove potential dye biases more effectively than a global normalization. After normalization, the noise level of each channel of an array chip was estimated by the median intensity of control spots (plant genes and buffer-only spots). Any normalized intensity below the estimated noise level in each channel was removed from our analysis. The preprocessed logarithmic intensity ratios were used to explore gene expression patterns and to identify significantly changed genes. A permutation test was used to assess the statistical significance of changes in the normalized log-transformed intensity ratios at each time point separately. Probabilities were adjusted by the false-discovery rate (FDR)[25] of <1% for multiple test correction, meaning that no more than 1% false-positive genes were in the significantly changed gene list at each time point. Finally, we further filtered out the genes with <1.5-fold changes, to achieve more stringent selection of significantly changed genes, in a manner similar to that reported previously.[26,27]

RESULTS

Effect of Corneal Trauma on Differential Gene Expression in the Lacrimal Gland

A total of 15,065 genes were evaluated over eight time points: 0.5, 1, 3, 8, 24, 72, 120, and 360 hours. Although the sequences

of these genes are known, not all of them have known functions.[28] According to the annotation from Stanford's Source database (http://source.stanford.edu; provided in the public domain by Stanford University, Stanford, CA), genes were divided into groups of known and unknown genes. There were 7814 known and 7251 unknown genes (51.9% and 48.1%, respectively). Of the 15,065 genes, 2,003 were filtered because their expression levels declined below the noise level, and a total of 13,062 were found to be expressed in the mouse lacrimal gland. We found that 3,799 genes, 29.1% of the total of 13,062 expressed in the lacrimal gland, showed statistical significance and at least a ±1.5-fold change at one or more of the eight time points after acute corneal injury. Of these, 1528 (40.2%) were known genes, and 2271 (59.8%) were unknown. Many genes had significantly altered expression at multiple time points. Counting each time point as a separate event, 56.9% of all significant changes in gene expression represented downregulation, and 43.1% represented upregulation. Any given gene may have contributed to either the downregulation or the upregulation groups. Of the known genes, 80.0% were primarily downregulated, and only 20.0% were upregulated, whereas of the unknown genes, 38.7% were downregulated and 61.3% were upregulated.

A longitudinal study generated a picture of the distribution of the known genes with significantly altered expression over all time points against time-matched controls. Corneal stimulation significantly induced long-lasting changes in gene expression over the whole time course, from 0.5 hour to 360 hours. Downregulation was dominant over upregulation at all time points in the known genes (Fig. 2). An overall profile of the time course of downregulated known genes showed that the changes in gene expression occurred as early as 0.5 hour, quickly reached the peak level at 1 hour, and then gradually recovered toward the normal level, although two smaller peaks appeared at 8 and 72 hours. The upregulated genes were always fewer and showed only one small peak at 1 hour (Fig. 2). The number of times that a gene is differentially expressed over the multiple time points may indicate the relative effect of corneal injury on the gene. Approximately 40% of the genes, among the 1528 known genes, were significantly changed at only one time point, whereas the remaining 60% were differentially expressed at more than one time point (Table 1; for a complete gene list, see Supplemental Table S1 at www.iovs.org/cgi/content/full/46/2/461/DC1).

Functional Classification of Known Genes Differentially Expressed

The 1528 known genes differentially expressed were classified into 19 functional groups (18 classified groups and 1 unclassified group) based on the annotation of genes used in our microarray chips[28] and Stanford's Source database. This classification is relative, however, since some genes can be included in more than one functional group due to their multiple functions. The number and percentage of genes in each group are shown in Figure 3, top panel, and the percentage of genes significantly up- or downregulated in each functional group is shown in Figure 3, bottom panel. In all functional groups except heat shock and growth factor, downregulation was more frequent than upregulation (Fig. 3, bottom panel). Predominant downregulation was found in the genes for house-

TABLE 1. Frequency and Number of Known Genes that Showed Altered Expression

	1 Time	2 Times	3 Times	4 Times	5 Times	6 Times	7 Times	8 Times	Total
Genes (*n*)	617	326	197	137	97	63	57	34	1528
Percentage	40.4	21.3	12.9	9.0	6.3	4.1	3.7	2.2	100
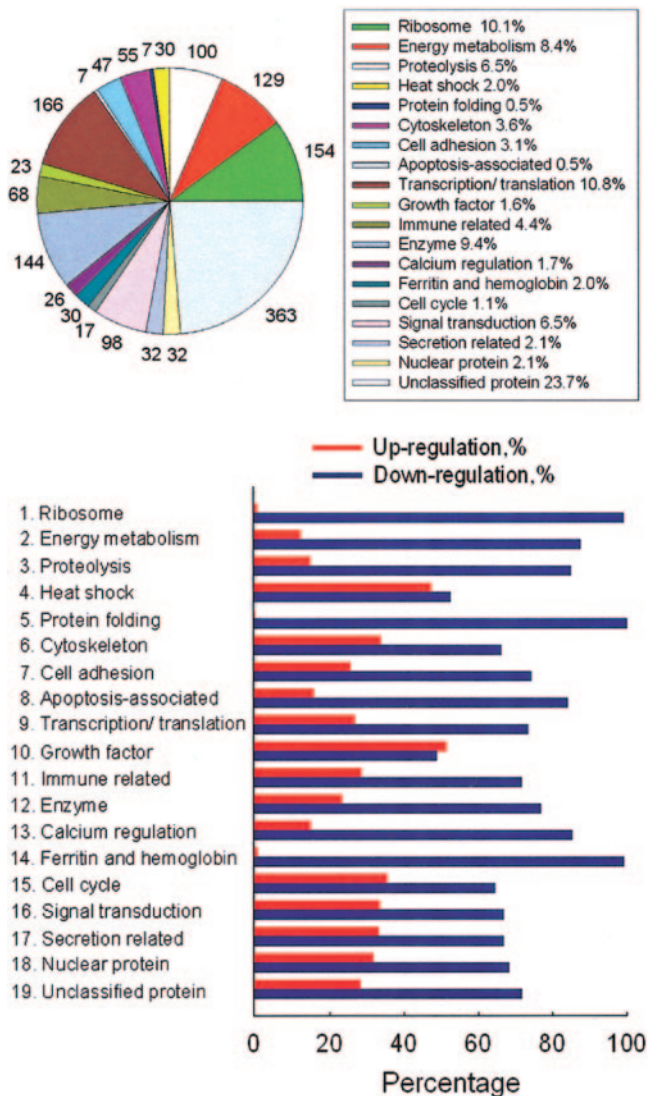
FIGURE 3. Functional classification of 1528 known genes with significantly altered expression and >1.5-fold change and a comparison of up- and downregulation within each functional group. The pie chart (*top left*) shows the proportion of each functional group and the total number of genes with altered expression in each group. The legend on the *top right* shows the names and percentages of the functional groups. The histogram (*bottom*) compares the percentage of (*red*) up- or (*blue*) downregulated genes within each functional group. In all groups, except heat shock and growth factor, more genes were downregulated than upregulated.

keeping (ribosome, cytoskeleton, and cell adhesion), energy metabolism, protein degradation (proteolysis), and protein folding and in apoptosis-associated genes. The genes for DNA and protein synthesis (transcription and translation), enzyme, immune-related proteins, cell cycle, signal transduction, and nuclear proteins were also strongly downregulated. The same result held true for genes presumably involved in tear secretion mechanisms and calcium regulation. Surprisingly, the ferritin and hemoglobin-related genes also predominantly showed downregulation (Fig. 3, bottom panel; for a complete gene list, see Supplemental Table S2 at www.iovs.org/cgi/content/full/46/2/461/DC1).

A more detailed analysis of the time course of each functional group continued to show predominant downregulation of almost all groups at nearly all time points (Fig. 4). Some of the functional groups at certain time points, however, were predominantly upregulated, such as the heat shock and growth factor groups. We found a total of 30 heat shock genes that had significantly changed expression at one or more time points with a unique pattern of relatively dynamic changes in expression levels over time (Figs. 5A–C). The heat shock genes were initially downregulated at 0.5 and 1 hour and were strongly upregulated at 8 hours, the peak of activity. At 24 and 72 hours, heat shock genes were downregulated and returned to nearly normal levels at 120 and 360 hours, indicating recovery from corneal trauma.

DISCUSSION

This study demonstrates that acute corneal injury induces, in vivo, long-lasting and widespread suppression of gene expression in the lacrimal gland. Overall, 56.9% genes were downregulated and 43.1% were upregulated of the 3799 genes with significantly changed expression. Moreover, of the known genes, 80.0% were downregulated and 20.0% were upregulated. The gene families that were suppressed involved almost all major functional groups, indicating a dramatic impact of corneal injury on cellular functions of the lacrimal gland.

Analysis of the gene families involved in the response of the lacrimal gland to corneal trauma indicates that many fundamental cell mechanisms were shut down by corneal injury. The significance of this mass suppression of major functional gene groups is not yet clear. Presumably, suppression of cell function is in some way protective. This strategy may be a method of conserving cell resources to make them available later. Suppression of such functional groups as housekeeping, energy metabolism, protein degradation, and DNA and protein synthesis indicates that this response occurs at a fundamental level and may have implications for nearly all cell functions. Suppression of the genes for apoptosis-related proteins, such as caspase 6, together with cytochrome c (energy metabolism group, see Supplemental Table S2 online), may also be an example of a conservative tactic, as this should lead to a slowdown in the application of apoptotic mechanisms.[29–32] Retarding apoptosis may allow the cells to repair or replace some damage to proteins and better tolerate the cellular stress of the lacrimal gland from overstimulation to the cornea, thus minimizing the number of cells that eventually succumb to apoptosis.[29,33]

Upregulation of heat shock genes was an unexpected result of our experiment, which indicated activation of the well-known stress response in vitro.[34] Activation of heat shock genes is the universal response to heat stress but also occurs after many other forms of stress such as arsenic poisoning, ischemia, and hypoxia.[35–37] It also occurs in vivo after strenuous exercise.[38,39] Our new finding is the initial suppression of heat shock genes at 0.5 and 1 hour and a striking activation to a peak at 8 hours after corneal injury (Fig. 5). The heat shock group comprises chaperone proteins that act to stabilize damaged cell proteins and permit a more orderly recovery from stress.[40–42] The significance of this early suppression of heat shock genes is unclear. Their subsequent activation, however, is consistent with other conservation measures to preserve cell resources and functions.[29–32] Later downregulation of heat shock genes at 24 and 72 hours (Fig. 5) may signal a shift to the activation of repair mechanisms that clear damaged proteins during recovery from the stress. At the last two time points, 120 and 360 hours, only a few heat shock genes had altered expression, either up or down (Fig. 5A), and the range became less dynamic compared with that at other time points (Fig. 5B). This may indicate the end of the stress response. Further evidence is that the overall number of genes with altered expression decreased dramatically at the last two time points (Fig. 2).
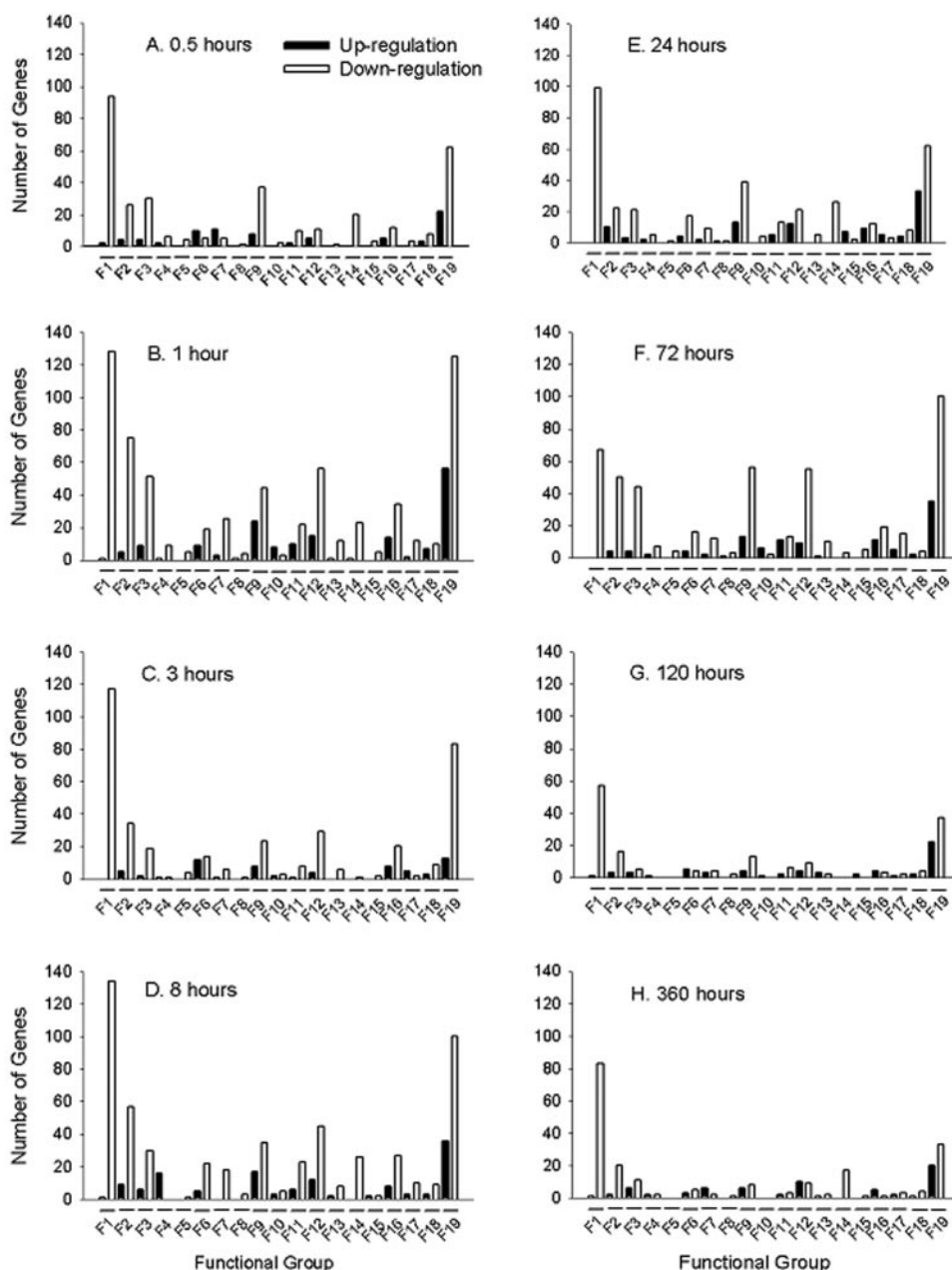
FIGURE 4. Number of genes significantly up- and downregulated in each functional group at each of the eight time points (0.5–360 hours, **A–H**). Downregulation predominated in most functional groups from 0.5 to 72 hours (**A–F**). The primary exceptions were the heat shock (F4) at 8 hours, growth factor (F10) at 1 and 72 hours, cytoskeleton (F6) and cell adhesion (F7) at 0.5 hour, and cell cycle (F15) and secretion-related (F17) at 24 hours. At (**G**) 120 and (**H**) 360 hours the number of genes decreased in most functional groups except ribosome (F1) and unclassified protein (F19).

The suppression of multiple genes for ferritin and hemoglobin was an unexpected finding for which, as yet, we have no satisfactory explanation. These genes are not thought to be active in lacrimal gland functions. Their general suppression may simply indicate very extensive gene suppression after corneal injury. It is likewise possible that these genes play some as yet undiscovered role in cell metabolism of the lacrimal gland, considering the multiple functions of hemoglobin[43] besides its basic function of oxygen transport in erythrocytes.

The genes involved in the regulation of tear secretion and associated calcium functions were also shut down in our study. For example, calcium is an important factor in tear secretion as a second messenger activated by neuronal input through cholinergic M3 muscarinic receptor, adrenergic $\alpha 1$ receptor, and vasoactive intestinal peptide (VIP) receptor.[3] Several proteins modulate calcium activity including annexin,[44] calcium binding protein P22,[45] voltage-dependent calcium channel,[46] calmodulin,[47] and calponin.[48] Almost all of them primarily showed downregulation in this study, indicating decreased

calcium activity (see Supplemental Table S2 online, group 13, calcium regulation). We found that some other related genes, such as secretory carrier member proteins 3 and 4, were also downregulated, although secretory carrier member protein 1 was upregulated (Supplemental Table S2, group 17, secretion related). Some genes for proteins secreted by the lacrimal epithelium were also primarily downregulated, such as lysozyme (Supplemental Table S2, group 3, proteolysis) and transforming growth factor (Supplemental Table S2, group 10, growth factor). This suggests that overall tear secretion activity is low. This finding was surprising, because it seems intuitively that the lacrimal gland cells should have been stimulated to produce more rather than less tearing in response to eye pain. The suppression of secretion-related gene expression further supports the concept that nearly all cell systems were shut down and brought to resting status to protect the cells from the damage produced by overstimulation. This finding is consistent with a phenomenon that has been observed in ophthalmic clinical practice for many years. Creation or exacerbation
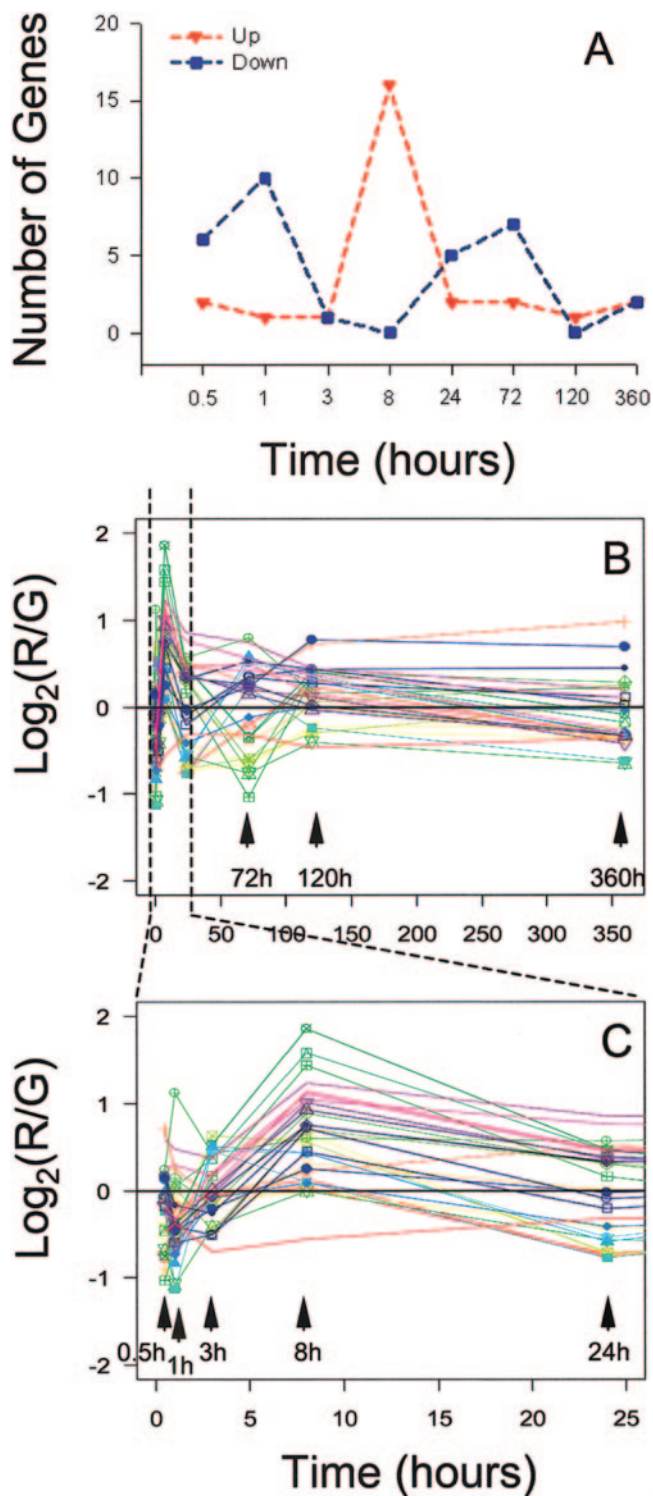
FIGURE 5. Time course of up- and downregulation of heat shock genes with significantly altered expression and quantitative gene expression levels in real time at all time points, including all 30 heat shock genes. (**A**) The curves show that heat shock genes with altered expression and >1.5-fold change were initially downregulated (*blue line*) at 0.5 and 1 hour and then were strikingly upregulated (*red line*) at 8 hours. Downregulation was predominant again at 24 and 72 hours. (**B, C**) The quantitative gene expression levels are plotted as values of $\log_2$ (R/G). A positive value (above the reference line, which is through the zero point) indicates upregulation, and a negative value (below the reference line) indicates downregulation. R (*red*) represents Cy5-tyramine labeling in the treated animals; G (*green*) represents Cy3-tyra-

of dry eye is quite common after cornea trauma, such as laser in situ keratomileusis (LASIK) corneal surgery or wearing contact lenses to correct vision defects.[4,9,10,49] Tear production was found to decrease after LASIK.[8,11] Suppression of tear production is an alternative mechanism that may occur as a direct result of corneal overstimulation and not as a consequence of LASIK-induced corneal denervation, as previously considered.[50] The cornea-to-lacrimal gland feedback mechanism has been proposed as a cause for the dry eye that occurs after LASIK procedures and other forms of corneal trauma.[2–4] Our finding that corneal injury induces widespread gene suppression in the lacrimal gland may provide genetic evidence to support this mechanism.

Our finding of overwhelming downregulation of gene expression in the lacrimal gland after corneal trauma indicates that lacrimal gland function has been shut down, at least temporarily. One may raise the question of whether such a change is a direct result of corneal trauma through neuronal input or an indirect outcome from an inflammatory response after corneal wounding. There is evidence that inflammation plays a role in lacrimal gland deficiency. For example, in some autoimmune diseases such as Sjögren syndrome, inflammation causes the dysfunction of the lacrimal gland.[3,51] However, more commonly, inflammation on the ocular surface is a consequence of damage to the lacrimal gland–corneal functional unit after corneal injury, and ocular surface inflammation potentiates the failure of the whole unit.[51] We found that many proinflammatory genes were significantly altered, either up- or downregulated, although we could not determine whether they were produced from the lacrimal gland epithelial cells or from the inflammatory cells that may have infiltrated the lacrimal gland tissue after corneal injury. The overall result showed that among the 68 inflammation-associated genes altered, 23 (33.8%) were upregulated, 38 (55.9%) were downregulated, and 7 (10.3%) showed both up- and downregulation over the time course. Counting the accumulated events in terms of changes in gene expression at any time point over the whole time course, we found 137 occurrences of changed expression. Among these changes, 39 (28.5%) were upregulation and 98 (71.5%) were downregulation (Supplemental Table S2, group 11, immune related). This result suggests that inflammation may not be a major process in the lacrimal gland after acute corneal injury.

Gene expression in the lacrimal gland after complete surgical parasympathetic nerve denervation has recently been investigated in the rat.[52] The response was assessed at the seventh day after surgical denervation. It is not surprising that complete denervation of the lacrimal gland causes decreased tear production and dry eye. However, the gene response in the lacrimal gland to denervation also differed from that which occurred after acute corneal injury. It appears that different mechanisms are operating during the lacrimal gland's response to the complete parasympathetic nerve denervation of the gland compared with mechanisms that come into play after corneal injury.

Investigation of the time course and extent of altered gene expression suggests clues to the mechanisms that may be responsible for these effects. The changes in gene expression occurred as early as 0.5 hour, quickly reached their maximum at 1 hour, attained two smaller peaks at 8 and 72 hours, and then declined to low levels of activity at later time points (Fig. 2). This may indicate a two-phase process: a relatively rapid early response mediated through neuronal pathways and a

mine labeling in the control animals. (**B**) The entire time course over 360 hours; (**C**) an expanded time scale from 0 to 25 hours. *Arrows:* time points when samples were collected.

later slow response, possibly mediated through a combination of neuronal and hormonal pathways. A stress state may exist in this later phase, since heat shock genes, well-known to be stress-response genes, were strongly activated at 8 hours after being downregulated during the early phase. Although our model consisted of an acute, single corneal injury in this study, this stimulus was not transient, because there was persistent damage to the cornea. Corneal injury is associated with greater pain than other causes such as iritis or conjunctivitis.[53] Such severe pain can lead to activation of the sympathetic nervous system as well as the pituitary–adrenal endocrine system,[53] which also indicates creation of a stress response. During stress, the circulating levels of melanocortin peptides: adreno-corticotropic hormone (ACTH) and α-melanocyte-stimulating hormone (α-MSH) are increased dramatically.[1,54] ACTH and α-MSH induce protein secretion[55–57] and phosphorylation[55] in the lacrimal gland. There is evidence for specific receptor binding sites of melanocortin receptors in the lacrimal gland.[58–60] The studies using molecular biology confirmed that lacrimal gland expresses melanocortin 5 receptor (MC5 R).[57,61] The hypothalamic-pituitary-exocrine axis has been proposed by Chen et al.[57] as a mechanism for stress-related exocrine effects after their study of MC5 receptor–deficient mice. However, the specific role of ACTH/α-MSH in gene regulation in the lacrimal gland after corneal injury remains to be elucidated in the future studies. Other hormones may also play a role. We acknowledge that female mice may have hormone mechanisms that are more variable than male mice.

One can expect that the gene expression pattern would be different with different degrees of corneal injury, because corneal nerves are functionally heterogenous and are able to distinguish different types of stimulation or corneal injury.[62] For example, corneal mechanical scratch, a jet airflow injury, or simple corneal physical pressure may produce a different gene expression pattern in the lacrimal gland, because the degree of corneal nerve stimulation and/or the degree of induced stress varies from one injury to another. We are unable to make a comparison of microarray data for different forms of corneal injury because of the unavailability of such information in the literature. Therefore, this topic is worth further investigation.

Another possible mechanism for cornea-lacrimal gland interaction may be the direct effect of the cytokines generated from the corneal wound. The mouse model in our study has been used by different investigators,[18,19] whose studies showed that the corneal chemical burn induces corneal epithelial and endothelial cell loss[18] and a large amount of inflammatory cell infiltration into the corneal stroma[18,19]—mostly of neutrophils and macrophages.[18] These inflammatory cells can be detected in the cornea at 2 days after corneal cauterization, with maximum concentration at 4 and 7 days after cauterization,[19] indicating that the inflammatory cells infiltrate the cornea over time. Inflammatory cells within the cornea can produce cytokines including interleukin (IL)-1 and tumor necrosis factor (TNF)-α,[63] which have direct influence on the corneal inflammatory process and neovascularization in the cornea.[18,64] However, the cytokines secreted from these inflammatory cells probably did not have direct effects on gene expression in the lacrimal gland, because the changes in gene expression of the lacrimal gland reached a peak very early, at 1 hour, showed two smaller peaks at 8 and 72 hours, and returned to nearly normal levels at 120 hours (5 days), whereas the inflammatory cell infiltration in the cornea reached its maximum at 4 and 7 days, when the changes in lacrimal gland gene expression were well past their peak. The damaged corneal epithelia cells themselves probably secreted cytokines including IL-1, platelet-derived growth factor (PDGF), and TNF immediately after corneal injury.[65] These cytokines penetrate the stroma and initiate the corneal wound-healing cascade.[65] However, whether the cytokines secreted from the corneal epithelium or keratocytes actually diffuse to the lacrimal gland by some means and modulate the gene expression of the gland seems doubtful, because of the lack of evidence for this mechanism.

In summary, using a mouse model and cDNA microarray technology, we found that acute corneal injury caused long-lasting and widespread gene suppression in multiple gene families in the mouse lacrimal gland. The time course of the gene expression indicated a possible two-phase process: initial regulation, probably mediated through stimulation of the cornea–brain–lacrimal gland reflex, and later regulation, possibly mediated through a combination of neuronal and hormonal mechanisms. The activation of heat shock genes at 8 hours after corneal injury indicates the initiation of a stress response in the mice. The results show that corneal injury has significant effects on gene regulation of the lacrimal gland, which may constitute genetic evidence to support the cornea–lacrimal gland feedback mechanism proposed for dry eye.

In addition, the results obtained from the present study display a sweeping picture of the underling genetic response of the lacrimal gland to corneal trauma. It supplies, for the first time, basic information concerning the genome-wide gene expression profile of the lacrimal gland associated with corneal wounding. These data generate opportunities to explore the interactions between organs, particularly the function of the lacrimal gland in the abnormal condition of corneal-injury-induced stress. Microarray studies facilitate the identification of potential target genes that are critical to the whole process of the stress response and other candidate genes that are essential in controlling lacrimal gland tear production and secretion. At the same time, however, the data from microarray analyses also present difficult challenges and questions of how to interpret the results. We do not as yet understand which cellular pathways are responsible for the process of gene regulation of the lacrimal gland in response to corneal injury or overstimulation. The microarray experiment itself has limited ability to answer these questions, which remain beyond the scope of the present investigation. Addressing these questions more completely necessitates further experiments at various levels: systemic, cellular, intracellular second messenger, nuclear transcription factor, DNA/RNA molecular, and protein, toward which our future research efforts are directed. We hope this study can serve as a model for this process and will attract the attention of the broader scientific community as well.

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