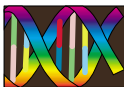
Microarray Analysis of Gene Expression in Human Donor Corneas



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Objectives: To use microarray analysis to identify genes expressed in human donor corneas and to create a preliminary, comprehensive database of human corneal gene expression.

Methods: A complementary DNA (cDNA) library was constructed from transplant-quality, human donor corneas. Biotin-labeled RNA was transcribed from the cDNA library and hybridized in duplicate to microarrays containing approximately 5600 human genes. Results were analyzed using a gene database of the National Institutes of Health, Bethesda, Md. Reverse transcriptase polymerase chain reaction analysis was performed to confirm corneal expression of genes identified by microarray analysis.

Results: Duplicate microarrays identified the expression of 1200 genes in human donor corneas. Chromosomal loci had been assigned to 1025 (85%) of these genes. A preliminary database of human corneal gene expression was compiled. A Web site containing these genes was created. Six collagen genes were identified that had not previously been localized within the cornea. Five apoptosis-related genes were identified, 4 of which had not previously been localized within the cornea. Three genes previously shown to cause corneal diseases were identified. Reverse transcriptase polymerase chain reaction analysis of genes identified by microarray analysis confirmed the corneal expression of 2 apoptosis-related genes and 1 collagen gene.

Conclusions: Microarray analysis of healthy human donor corneas has produced a preliminary, comprehensive database of corneal gene expression. Large-scale analysis of gene expression has the potential to generate large amounts of data, which should be made readily accessible to the scientific community. The Internet offers many potential advantages as a medium for the maintenance of these large data sets.

Clinical Relevance: Identification of structural, apoptosis-related, and disease-causing genes within the cornea by microarrays may increase the understanding of normal and abnormal corneal function with likely relevance to corneal diseases and transplants.

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FUNDAMENTAL to understanding normal tissue function is a global knowledge of the thousands of genes expressed in the various cell types that comprise that tissue. This baseline knowledge should facilitate the identification of alterations from normal gene expression that play important roles in disease pathogenesis. Efforts to comprehensively study gene expression patterns in normal tissues and altered gene expression patterns in diseased tissues will require a complete knowledge of the human genetic sequence and methods to accurately and simultaneously analyze large amounts of genetic information. Both of these requirements have recently become available through the ongoing progress of the Human Genome Project and breakthroughs in high-efficiency genetic analysis techniques such as DNA microarrays.

DNA microarrays are a new and powerful technique to study the expression of thousands of genes in a single experiment.[1] A microarray is a solid substrate such as a glass slide or nylon membrane to which known, single-stranded DNA molecules are attached at distinct locations. The density of these locations on a microarray can reach upwards of 250 000/cm^2, as demonstrated by van Hal et al.[2] Experimental messenger RNA (mRNA) is labeled as a complex mixture and exposed to the microarray. Labeled mRNA molecules will bind to complementary sequences on the microarray and can be detected in a semiquantitative manner using automated techniques. Advantages of DNA microarrays include simultaneous screening for the expression of large numbers of

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MATERIALS AND METHODS

Twenty corneal-scleral rims were obtained at the time of penetrating keratoplasty, and peripheral corneal tissue was carefully dissected, placed in microcentrifuge tubes, and immediately stored at –80°C. Two entire transplant-quality donor corneal buttons were placed in microcentrifuge tubes and immediately stored at –80°C. The death to preservation time of all tissues used in this study was less than 12 hours. All tissues used in this study were obtained from donors younger than age 65 years. A complementary DNA (cDNA) library was constructed using standard methods from the pooled corneal tissues described.[8] The number of clones contained in the primary cDNA library was estimated to be 1.0×10^6.

Standard methods were used to recover phagemids by mass excision protocol (pBluescript; Stratagene, La Jolla, Calif). The number of plasmids excised was 1.6×10^6. The ratio of clones excised to the number of independent clones in the library was 1.6:1. Excised clones were used to transfect a large-volume cell culture (SOLR; Stratagene, and plasmid "maxi-preps" were performed with a standard kit and protocol (Qiagen, Valencia, Calif). Plasmids were digested using restriction endonuclease (NotI; Life Technologies, Rockville, Md), phenol-chloroform extracted, and ethanol precipitated.

Biotin-labeled cRNA molecules were produced by in vitro transcription (Enzo Diagnostics, Farmingdale, NY), digested with DNase I (Life Technologies), and purified using commercially available spin columns (RNeasy; Qiagen). Analysis of biotin-labeled cRNA using the HuGeneFL microarray (Affymetrix, Santa Clara, Calif) was performed in duplicate (Research Genetics, Huntsville, Ala) by hybridizing the same labeled cRNA sample to 2 identical microarrays within a 6-week time period.

Microarray analysis results were analyzed using GenBank[9] and LocusLink,[10] online genetic databases sponsored by the National Library of Medicine of the National Institutes of Health, Bethesda, Md. A corneal genetics web site was created using the y-Base Informatics Engine (y-DNA Inc, Palo Alto, Calif).

Total RNA was extracted using TRIZOL reagent (Life Technologies) from 2 pooled, whole, transplant-quality donor corneas. Reverse transcriptase polymerase chain reaction (RTPCR) was performed using standard methods (Applied Biosystems, Foster City, Calif). One microgram of total RNA was used as template for first-strand cDNA synthesis. Polymerase chain reaction (PCR) was performed using 5 µL of each cDNA sample in a final reaction volume of 100 µL. A final concentration of 2.5 µM was used for each PCR primer. The PCR cycling conditions included an initial denaturation for 105 seconds at 95°C, followed by 35 cycles of denaturation for 15 seconds at 95°C, annealing for 30 seconds at 60°C, and extension for 7 minutes at 72°C. The PCR primers for α1 type IV collagen included (sense) 5′-CAAGTTCAGCACAATGCCCTTC-3′ and (antisense) 5′-AATGGTCTGGCTGTGCACGGC-3′. The predicted PCR fragment corresponds to nucleotide positions +141 to +351 for an overall length of 211 base pairs (bp).[11] The PCR primers for caspase 7 included (sense) 5′-ATGGCAGATGATCACGGCTGTATTG-3′ and (antisense) 5′-TATAGACAATCACGTCAAAACCCA-3′. The predicted PCR fragment corresponds to nucleotide positions +44 to +377 for an overall length of 334 bp.[12] The PCR primers for TRAIL (tumor necrosis factor–related apoptosis-inducing ligand) included (sense) 5′-GAAGGAAGGGCTTCAGTGACCGG-3′ and (antisense) 5′-CTAACGAGCTGACGGAGTTGC-3′. The predicted PCR fragment corresponds to nucleotide positions +33 to +361 for an overall length of 329 bp.[13] The RTPCR products were visualized after dilution (caspase 7, 1:3; TRAIL, 1:10; and α1 collagen IV, 1:5), electrophoresis in 1.2% agarose gels, and staining with 1-µg/mL ethidium bromide.

genes, the ability to use small amounts of starting material, and mass production, which enables standardized, comparative analysis between samples.[2]

The acceptance of this technology is growing rapidly as microarrays are being used in an increasing number of experimental applications. These include analysis of gene expression in normal embryonic development[3] and pathologic states such as breast cancer[4] and myocardial infarction.[5] Microarrays also have been used to identify novel genes expressed in brain tissue[6] and for positional cloning of a disease gene in Niemann-Pick disease, type C.[7]

Given the successful use of microarray analysis in other biological systems, we sought to apply this technique to study gene expression in human donor corneal tissue. Such analysis may be useful for understanding the genetic basis of normal corneal function as well as corneal disease processes such as graft failure, inflammation, degenerations, and dystrophies. Knowledge of what genes are or are not expressed in a given corneal disorder could lead to new and definitive treatment strategies, including interventional drugs and gene therapies. These strategies may be particularly relevant and feasible for the cornea because the tissue is relatively less complex, can be manipulated ex vivo, and can be easily assessed visually.

Microarray analysis is a feasible method to begin compiling a comprehensive database of genes expressed in human corneas. Such a database of genes may have broad applications for corneal genetics research. Any comprehensive database of corneal genes would be expected to be relatively large and to grow as more genes are identified in this tissue and as the assembly phase of the Human Genome Project defines novel genes from currently available sequence information. Such a large database would be most useful if it could be readily updated, freely accessible to the global research community, and effectively interfaced with preexisting gene databases. Given these desirable features, an Internet Web site could be an ideal format for a comprehensive corneal gene database. Such a Web site could potentially enhance the progress of corneal genetics research by increasing the accessibility of relevant genetic information and facilitating discussion among corneal genetics researchers. As the proposed comprehensive corneal gene database grows and becomes more clinically relevant, it also could serve as a model for similar efforts in other clinical disciplines.

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Table 1. Chromosome Loci of Genes Identified in Human Donor Corneas by Microarray Analysis*

Chromosome	No. of Corneal Genes Identified
1	118
2	70
3	45
4	37
5	42
6	67
7	53
8	35
9	29
10	44
11	67
12	69
13	23
14	40
15	33
16	28
17	48
18	17
19	48
20	23
21	17
22	29
X	42
Y	1
Unmapped†	175

*Chromosome loci of confirmed corneal genes were determined using GenBank[9] and LocusLink.[10]
†Chromosome loci not assigned in GenBank[9] or LocusLink.[10]

Table 2. Detection of Collagen Gene Expression by MIcroarray Analysis of Human Donor Corneas*

Collagen Type	Chromosome Locus	GenBank[9] Accession No.	Associated Disease	Reference
COL4A1	13q34	M26576	. . .	14
COL11A1	1p21	J04177	Stickler syndrome	15
COL16A1	1p34-p35	M92642	. . .	16
COL5A2	2q14-q32	M11718	Ehlers-Danlos syndrome type II	17
COL4A3	2q36-q37	M81379	Alport syndrome	18
COL6A3	2q37	X52022	Bethlem myopathy	19

*Ellipses indicate no associated disease reported.

Table 3. Detection of Apoptosis-Related Gene Expression in Human Donor Corneas by Microarray Analysis

Gene Name*	Chromosome Locus	GenBank[9] Accession No.	Reference
CLARP	2q33-q34	AF005775	21
TRAIL	3q26	U37518	22, 23
Bcl-xL	20	Z23115	24, 25
Bcl2/p53 binding protein (BBP/53BP2)	1q42.1	U58334	26-29
Caspase 7	10q25	NM001227	30, 31

*CLARP indicates caspase-like apoptosis regulatory protein 2; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand.

RESULTS

Microarray analysis of a cDNA library constructed from transplant-quality human donor corneas was performed in duplicate. The first microarray identified the expression of 1794 human genes. The second microarray identified the expression of 1406 human genes. A total of 1200 shared genes were identified on both microarrays. The concordance rate between microarrays was 67%, calculated as the number of shared genes identified on both microarrays (1200) divided by the larger number of genes identified on a single microarray (1794). Only the 1200 genes confirmed by both microarrays as expressed in the cornea were used in subsequent analyses in this study. As the microarrays used in both experiments contained approximately 5600 human genes, the 1200 genes with confirmed corneal expression represent approximately 22% of the total genes contained on the microarrays.

The 1200 genes with confirmed corneal expression were analyzed using GenBank[9] and LocusLink.[10] Of the 1200 confirmed corneal genes, 1025 (85%) had assigned chromosomal loci in GenBank or LocusLink (**Table 1**).[14-19] Thus, 175 confirmed corneal genes (15%) had unassigned chromosomal loci in GenBank or LocusLink (Table 1).

The 1200 genes with confirmed corneal expression were used as the basis of a corneal genetics Web site named CorneaNet.[20] CorneaNet includes the 1200 genes identified in the present study in addition to 53 genes identified from the literature as being expressed in human corneas or cultured human corneal cell lines. Each entry in CorneaNet includes a gene name, symbol, chromosome locus, and GenBank accession number with an active link to the GenBank entry for the specified gene. CorneaNet is open to contributions from the research community and is updated regularly with genes newly reported in the literature as being expressed in human corneal tissues.

Six types of collagen subunits were included among the confirmed corneal genes identified by microarray analysis (**Table 2**). These included α1 type IV, α1 type XI, α1 type XVI, α2 type V, α3 type IV, and α3 type VI collagens (Table 2). Mutations in α1 type XI collagen cause Stickler syndrome with a "beaded" type 2 vitreous phenotype.[15] Mutations in α2 type V collagen cause Ehlers-Danlos syndrome type II.[17] Mutations in α3 type IV collagen cause autosomal recessive Alport syndrome.[18] Mutations in α3 type VI collagen cause Bethlem myopathy.[19] None of these 6 collagen subunits has previously been identified in human corneas.

Five apoptosis-related genes were included among the confirmed corneal genes identified by microarray analysis (**Table 3**).[21-31] These included caspase-like apoptosis regulatory protein 2, TRAIL, Bcl-xL, Bcl2/p53-binding protein (BBP/53BP2), and caspase 7 (Table 3). Caspase-like apoptosis regulatory protein 2 is a protein with a homologous sequence to caspase 8 and caspase 10 that may stimulate apoptosis through regulatory effects on caspase 8.[21] The TRAIL is a member of the tumor necrosis factor family that can induce apoptosis of activated T lymphocytes.[22,23] Bcl-xL is an inhibitor of apoptosis that is often overexpressed in solid tumors and shares sequence homology with Bcl-2, an-

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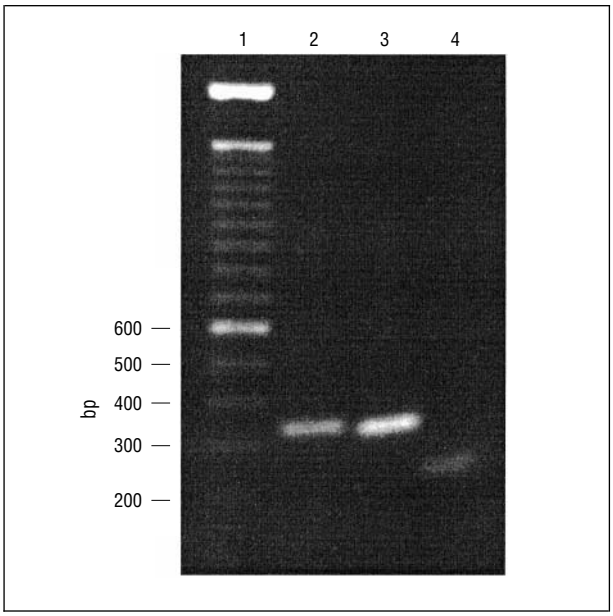
Table 4. Detection of Genes Causing Corneal Diseases by Microarray Analysis of Human Donor Corneas

Gene Name	Chromosome Locus	GenBank[9] Accession No.	Corneal Disease(s)	Reference
Transforming growth factor β–induced gene product (*BIGH3,* keratoepithelin)	5q31	M77349	Granular dystrophy I, lattice dystrophies I and IIIA, Avellino dystrophy, Reis-Bucklers dystrophy, Thiel-Behnke dystrophy	33, 34
Keratin 12 (*KRT12*)	17q12	D78367	Meesmann dystrophy	34-36
PAX6	11p13	M93650	Peters anomaly, autosomal dominant keratitis	37-39



Reverse transcriptase polymerase chain reaction analysis of human corneal total RNA. Lane 1, DNA size ladder; lane 2, caspase 7 (334 base pairs [bp]); lane 3, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) (329 bp); lane 4, α1 collagen IV (211 bp).

other apoptosis inhibitor.[24,25] BBP/53BP2 is a proapoptotic protein that interacts with p53, Bcl-2, and the p65 subunit of NF-kappaB[26,27] and is overexpressed in lung cancer cell lines and in vitro cell lines exposed to UV stress.[28,29] Caspase 7 is a proapoptotic cysteine protease that induces massive apoptosis when overexpressed in human prostate cell lines.[30] Selective inhibition of caspase 7 prevents apoptosis and maintains cell functionality.[31] Of these 5 genes, only Bcl-xL has previously been identified in human corneal cells.[32]

Three corneal disease–causing genes were included among the confirmed corneal genes identified by microarray analysis (**Table 4**).[33-39] These included keratoepithelin (*BIGH3*), keratin 12 (*KRT12*), and *PAX6* (Table 4). Numerous mutations in the *BIGH3* gene produce an abnormal protein that accumulates in the cornea and produces granular dystrophy I, lattice dystrophies I and IIIA, Avellino dystrophy, Reis-Bucklers dystrophy, and Thiel-Behnke dystrophy.[33,34] Six mutations in the keratin 12 gene have been demonstrated to cause Meesmann dystrophy.[34-36] Mutations in the *PAX6* gene cause anterior segment abnormalities such as aniridia and Peter anomaly, as well as autosomal-dominant keratitis.[37-39]

Three genes, caspase 7, TRAIL, and α1 collagen IV, were selected at random for RTPCR analysis to confirm corneal expression as determined by microarray analysis. Specific amplification products of the expected sizes were detected for all 3 genes (**Figure**).

COMMENT

The recent completion of the sequencing phase of the Human Genome Project provides a wealth of genetic information that should facilitate clinically relevant studies of normal and abnormal cellular processes. One potentially useful application of this information is the creation of comprehensive databases of genes expressed in a given normal or abnormal tissue or cell type. An initial attempt to investigate quantitative and qualitative aspects of gene expression in the corneal epithelium was performed using the conventional technique of sequencing 1069 randomly selected cDNA clones.[40] A similar study reported the sequencing of 1060 cDNA clones from a human trabecular meshwork cDNA library.[41]

DNA microarrays represent a powerful technique to screen large amounts of genetic material for known sequences. This method has been used in ophthalmology to study alterations in gene expression caused by the photoreceptor homeobox gene *CRX*[42] and elevations in intraocular pressure.[43] In the present study, this technique was used to identify the expression of 1200 known genes in transplant-quality human donor corneas. Only those genes positively identified on 2 identical microarrays were included in this study with a concordance rate of 67%. This conservative approach was followed to minimize the possibility of false-positive genes. Further studies are in progress to confirm corneal expression for the genes identified by only a single microarray in these experiments.

As this study exemplifies, current techniques of genetic analysis can generate extremely large amounts of information in a single experiment. Disseminating this information via the Internet offers the advantages of being easily accessible and modifiable. Furthermore, the Internet allows such gene databases to interface with preexisting, high-quality, and authoritative online genetic Web sites such as GenBank[9] and Online Mendelian Inheritance in Man.[44] This approach was used in the creation of CorneaNet,[20] which may become a useful resource for the cornea research community by improving the dissemination of genetic information. If successful, CorneaNet may serve as a model for online databases of gene expression in other tissues.

One limitation of microarray analysis is the inability to identify previously unreported genes. However, the ongoing assembly phase of the Human Genome Project

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Table 5. Genes Expressed in Human Donor Corneas That Map to Chromosome Loci Associated With Corneal Dystrophies

Corneal Dystrophy	Inheritance*	Chromosome Locus	No. of Corneal Genes at Locus	Reference
Central crystalline	AD	1p36	12	45
Posterior polymorphous	AD	20q11	2	46
Congenital hereditary endothelial I	AD	20p	7	47
Congenital hereditary endothelial II	AR	20 tel	2	48
Keratoconus	AD	21q21.1-q21.1	2	49
X-linked megalocornea	XR	Xq12-q26	16	50

*AD indicates autosomal dominant; AR, autosomal recessive; and XR, X-linked recessive.

should identify all of the estimated 30 000 genes in the human genome. This information, combined with continuing advances in microarray construction, should yield full-genome microarrays in the near future. Such tools should greatly facilitate the development of truly comprehensive gene expression databases.

Microarray analysis is an efficient way to investigate the genetic basis of normal and abnormal biological processes.[3-7] In this study, microarray analysis identified corneal expression of 6 collagen genes, none of which had previously been localized to this tissue. These results may lead to a more sophisticated understanding of the contributions of various collagens to the structural integrity of the corneal stroma. Similarly, the expression of 5 apoptosis-related genes were identified in the donor-quality corneas used in this study. Four of these genes had not been previously localized to the cornea. These results may provide insights into the possible role of apoptosis in the ultimate success or failure of corneal grafts. Caspase 7 is a powerful initiator of apoptosis,[30] and potent inhibitors of its activity have been shown to block caspase 7–mediated apoptosis.[31] Such inhibitors could ultimately be useful additives to corneal storage solutions to improve the viability of donor corneas.

Much progress has been made recently in identifying genes causing corneal dystrophies.[34] Several corneal dystrophies such as central crystalline dystrophy, posterior polymorphous dystrophy, congenital hereditary endothelial dystrophies I and II, keratoconus, and X-linked megalocornea have been mapped to chromosome loci but await identification of causative genes (**Table 5**).[45-50] The search for these disease-causing genes might be facilitated by knowledge of which genes present at specific chromosome loci are expressed in the cornea. Thus, the database of corneal genes identified by microarray analysis includes chromosome loci when available.

The present study is the first to our knowledge to apply microarray analysis to study corneal gene expression. The results were used to create a preliminary, online database of genes expressed in normal donor corneas. Microarray analyses of corneal ulcers, dystrophies, graft rejection, and others may provide insights into the genetic bases of these pathologic processes that may, in turn, lead to better treatments for corneal diseases.

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