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Microarray and protein analysis of human pterygium

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Purpose: Pterygium is a sunlight-related, ocular-surface lesion that can obscure vision. In order to identify specific genes that may play a role in pterygium pathogenesis, we analyzed the global gene expression profile of pterygium in relation to autologous conjunctiva.

Methods: Oligonucleotide microarray hybridization was used to compare the gene expression profile between human whole pterygium and autologous conjunctiva. Selected genes were further characterized by RT-PCR, western blot, and immunohistochemistry, and comparisons were made with limbal and corneal tissues.

Results: Thirty-four genes exhibited a 2 fold or greater difference in expression between human whole pterygium and autologous conjunctiva. Twenty-nine transcripts were increased and five transcripts were decreased in pterygium. Fibronectin, macrophage-inflammatory protein-4 (MIP-4), and lipocalin 2 (oncogene 24p3; NGAL) were increased 9, 5, and 2.4 fold, respectively, while Per1 and Ephrin-A1 were decreased 2 fold in pterygium. Western blots showed that fibronectin and MIP-4 were increased in pterygium compared to limbus, cornea, and conjunctiva. Immunohistochemical analysis showed fibronectin in the stroma; lipocalin 2 in the basal epithelial cells, basement membrane, and extracellular stroma; and MIP-4 in all areas of the pterygium.

Conclusions: These data show both novel and previously identified extracellular-matrix-related, proinflammatory, angiogenic, fibrogenic, and oncogenic genes expressed in human pterygium. Comparisons of selected genes with limbal and corneal tissues gave results similar to comparisons between pterygium and normal conjunctiva. The increased expression of lipocalin 2, which activates matrix metalloproteinases (MMP), is consistent with our previous findings that MMP-9 and other MMPs are highly expressed in pterygium basal epithelium.

Pterygium is a sunlight-related, ocular-surface disease that can obscure vision [1,2]. It occurs most frequently in populations located near the equator and in laborers who work outdoors or in specific factory environments [3]. There is a higher prevalence of pterygium in farmers, watermen, postal workers, sawmill workers, and welders. A cost of illness study in Australia showed that pterygium occurred in 12% of Australian men over 60 years of age [4], and a population-based survey conducted in rural southern China demonstrated a pterygium prevalence of 33% in subjects aged 50 years or older [5]. In the United States, pterygium is common in the southern and western states and in rural populations throughout the United States. In addition, watermen who work on the Chesapeake Bay in Maryland have a higher incidence of pterygium. For example, Taylor et al. [6] studied a group of watermen and found 140 pterygia and 642 pinguecula (a fibroblastic growth associated with pterygium pathogenesis) in 838 watermen.

Pterygium is a wing-shaped, epithelial-covered fibrovascular lesion that originates from the limbus, more often on the nasal than temporal side [1]. Visual impairment can result from astigmatism induced by the lesion even before involvement of the central cornea; progression of the lesion with migration centrally into the visual axis results in vision loss [7,8].

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The standard treatment for pterygium is surgical removal, which has a recurrence rate as high as 61-82% [8,9]. If the excision is combined with adjunct treatments, such as conjunctival autograft, amniotic membrane grafts, beta-radiation, or mitomycin C, recurrence can be reduced to 2-31%, depending on factors such as age, geographic location, occupation, pterygium morphology, and surgeon experience [8,10-12].

Numerous theories have been postulated for the pathogenesis of pterygia, including degeneration, ultraviolet irradiation, oxidative stress, immunological and anti-apoptotic mechanisms, and viral infections [13-19]. Epidemiological data strongly links sunlight exposure to the occurrence of pterygium, however, its etiology has not been fully explained.

To address this, a number of studies have focused on identifying proteins expressed in pterygium. Pro-inflammatory cytokines and angiogenic and fibrogenic growth factors have been reported in pterygium [1]. Several groups, including ours, have demonstrated the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in the epithelium overlaying the pterygium, in altered limbal basal epithelial cells and in cultured pterygium fibroblasts [20-22]. These findings demonstrate the dynamic and angiogenic nature of this lesion. Other genes, such as p53, have been reported to be overexpressed in pterygium [13,23,24], although lower levels and monoallelic deletions of p53 have also been reported [25,26].

In addition to studies on pterygium-associated proteins, a cDNA microarray study showed differential gene expression

in cultured pterygial fibroblasts compared to cultured normal conjunctival fibroblasts [27]. However, a global gene expression profile of fresh, surgically removed whole pterygium, which includes all component cell types, has not been reported. In the present, more extensive, oligonucleotide microarray study, we identified gene transcripts that are over- or underexpressed in pterygium when compared to normal conjunctiva, limbal, and corneal tissues. We report a number of new genes associated with pterygium, including lipocalin 2 and macrophage inflammatory protein-4 (MIP-4), and show that these proteins have different distributions within the pterygium tissue.

METHODS

The use of pterygium specimens was approved by the Institutional Review Board of Kaiser Permanente Medical Center, Sacramento, CA and specimens were handled in accordance with the Declaration of Helsinki. Whole pterygium, which includes both epithelial cells and stromal fibroblasts, and normal residual superior limbal conjunctiva graft tissues were surgically removed and, depending on the subsequent analysis, were immediately frozen, fixed in formalin, or placed in a solution of RNA*later* (Ambion, Austin, TX). Corneal and limbal tissues were dissected from donor eyes (NDRI, Philadelpha, PA) and prepared in the same manner.

RNA isolation: Total RNA was extracted from individual specimens with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). All RNA samples were treated with 20 U RNase-free DNase 1 (Boehringer Mannheim Biochemicals/ Roche Molecular Biochemicals, Indianapolis, IN) to eliminate any contaminating DNA. The concentration of the RNA was measured with a UV spectrophotometer (Shimadzu, Columbia, MD). The quality of the RNA was evaluated by running RNA samples on 2% E-gels (Invitrogen Life Technologies).

Microarray analysis: Total RNA (6 μg) from each of three whole pterygia (two primary and one recurrent) and three autologous conjunctiva was converted to cDNA using the SuperScript Choice System (Invitrogen Life Technologies) and a T7-(dT_{24}) primer (GENESET Corp., San Diego, CA). cDNA was purified using a phenol-chloroform-isoamyl alcohol extraction. The cDNA was used for the in vitro synthesis of biotin-labeled cRNA using the BioArray RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). cRNA was purified using RNeasy mini kits (Qiagen, Valencia, CA) and fragmented using a fragmentation buffer (200 mM Tris-acetate, pH 8.2, 500 mM potassium acetate, and 150 mM magnesium acetate). Labeled cRNA (10 μg) was hybridized for 16 h to Affymetrix human HG_U95Av2 chips, representing over 10,000 genes. Duplicate chips were used for each of the six individual samples. The GeneChips were washed and stained according to the manufacturer's recommendations (Affymetrix, Santa Clara, CA). Each chip was scanned using a confocal laser scanner after an initial staining with streptavidin phycoerythrin, and after a second antibody staining used to amplify the signal.

Data analysis was performed using Affymetrix Microarray Suite 5.0 software to generate an absolute analysis for each chip. The gene signal intensities were analyzed using GeneSpring software (Silicon Genetics, Redwood City, CA). A Welsh t-test was performed to find genes with a $p<0.05$. This gene list was then filtered to find genes with a 2 fold change between the composite data from pterygium and conjunctival tissue. Genes were clustered according to information available at the National Center for Biotechnology Information (NCBI), National Library of Medicine, NIH.

RT-PCR: Specific gene products were generated with the One Step RT-PCR system (Invitrogen Life Technologies). Primer sets, listed in Table 1, were designed from published human DNA sequences obtained from the NCBI. The linear range for each reaction was determined by varying the number of cycles and RNA concentrations. Subsequently, four different concentrations of total RNA template (Figure 1A) and three separate experimental sets of RNA were used for each gene. Control reactions with heat-inactivated reverse transcriptase were performed to confirm that the RNA was devoid of DNA contamination, and RT-PCR products were sequenced to verify the target gene (CEQ 2000, Beckman Coulter, Fullerton, CA). RT-PCR products were analyzed on 2% E-gels (Invitrogen Life Technologies). The gels were photographed (Syngene, Synoptics Ltd., Cambridge, UK), and the densities of the bands were determined using ImageQuant for Windows NT (Version 5.0) from Molecular Dynamics (Sunnyvale, CA). Mean values and standard deviations were calculated from three separate sets of primary pterygia and normal conjunctiva.

Western blot analysis: Proteins were extracted from primary whole pterygia and from specific regions of the cornea and limbus from donor eyes (NDRI) using T-PER (Pierce, Rockford, IL). Protein concentrations were determined (Bio-Rad Protein Assay, Bio-RAD Laboratories, Hercules, CA). Equal amounts of protein were loaded onto NuPage 3-8% Tris acetate gels for fibronectin and 12% Bis-tris gels for MIP-4 (Invitrogen Life Technologies). Proteins were electrotransferred to nitrocellulose membranes (Bio-Rad), and the blots were then incubated for 1 h in PBS/Tween containing 5% (w/v) nonfat milk powder. The blots were incubated overnight at 4 °C with primary antibodies; monoclonal antibodies for fibronectin (1:4,000; Accurate Chemical & Scientific Corporation, Westbury, NY) and monoclonal antibody for MIP-4 (1:500; Cell Sciences, Canton, MA). After overnight incubation, the blots were washed in PBS/Tween three times for 15 min and then incubated with a 1:60,000 dilution of peroxidase-labeled mouse antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) at 37 °C for 1 h. The proteins were visualized using Western Lightning Chemiluminescence Reagent™ (PerkinElmer Life Sciences, Inc., Boston, MA). Recombinant fibronectin (Sigma, St. Louis, MO) and MIP-4 (Chemicon, Temecula, CA) were used as standards.

Immunohistochemistry: All samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Immunohistochemical staining for fibronectin, lipocalin 2, MIP-4,

and lipocalin-type prostaglandin D synthase (L-PGDS) were performed as follows; four µm-thick sections were deparaffinized, treated with 3% hydrogen peroxide to block endogenous peroxidase activity and then treated with 10 mM citrate buffer, (pH 6.0) for 10 min at 100 °C, followed by cooling at room temperature for 20 min. Slides were then incubated with primary antibodies against fibronectin, (1:300, Chemicon), lipocalin 2 (1:250, R&D Systems, Inc., Minneapolis, MN), MIP-4 (1:100, Cell Sciences, Canton, MA), or lipocalin-type prostaglandin D synthase (1:250 Cayman Chemical, Ann Arbor, MI). Tissue sections were incubated with appropriate primary antibody overnight at 4 °C. After washing unbound primary antibody, sections were treated with commercial biotinylated secondary anti-immunoglobulin, followed by avidin coupled to biotinylated horseradish peroxidase, at room temperature, according to the manufacturer's instructions (LSAB2kit, DAKO Corporation, Carpinteria, CA). Immunohistochemical reactions were developed with diaminobenzidine as the chromogenic peroxidase substrate. Sections were counterstained with hematoxylin. No staining was observed in tissues without primary antibody.

RESULTS

Microarray analysis: Microarray analysis was performed on three separate whole pterygia (two primary and one recurrent) and three superior conjunctiva samples that had been surgically removed from the same patients. Thirty-four expressed genes, common to both primary and recurrent pterygium, demonstrated a 2 fold or greater difference between ptergium and autologous conjunctiva (Table 2). Twenty-nine genes were overexpressed and five genes were underexpressed in pterygium.

Upregulated genes included proteins associated with cell adhesion and migration, extracellular matrix, inflammation, angiogenesis, and oncogenesis. Fibronectin, a glycoprotein involved in cell adhesion and migration, was upregulated 9 fold at the mRNA level in pterygium compared to conjunctiva. MIP-4, a chemokine and T cell chemoattractant, was upregulated 5 fold. Two members of the lipocalin family, retinol binding protein 1 and lipocalin 2 (oncogene 24p3; NGAL), were increased by 3.6 and 2.4 fold, respectively.

Differentially expressed genes were clustered into functional categories (Table 3). The category having the greatest number of overexpressed genes is a cluster of cell adhesion

TABLE 1. PRIMERS USED FOR RT-PCR

Name	GenBank accession number	Primer sequence	Product (bp)
Fibronectin	X02761	F: CATGTCTCTCTGCCAAGATCCATCT R: TTGTTCCTACAGTATTGCGGGCCAG	390
Lipocalin 2	BC033089	F: CTGGCAGGGAATGCAATTCTCAGAG R: TCTCCCAGCTCCCTCAATGGTGTTC	490
Macrophage inflammatory protein-4 (MIP-4)	Y13710	F: ACTTCTCTGCCTGCCCAGCATCATG R: GACTCTTAGAAGAGGTGGCCTCCAG	402
GAPDH	NM_002046	F: CCACCCATGGCAAATTCCATGGCA R: CCACCTGGACTGGACGGCAGATCT	600
Carbonic Anhydrase	AK096880	F: CTTCAAATGAGGCTGCTGGATCTTG R: AAACTGGTGCCCAATAGCTAGCCAT	340
Chloride intracellular channel 2 (CliC2)	BC022305	F: GCAAACCACTGCAATCCTGAATGAC R: CATGAGGTGGTCATAGTTCTAGGCG	361
Ephrin-A1	NM_004428	F: CAGCTGAATGACTACGTGGACATCA R: TGGGTCATCTGCTGCAAGTCTCTTC	390
Period 1 (Per1)	AF022991	F: CAAGAGCACAAACTCTCAGAGCCCA R: GATGGAGCAGTGGAACCATAGAAG	550

The names, GenBank accession numbers, sequences, and product lengths for all gene-specific primers used in this study are listed. The entries in the "Primer sequence" column are for the forward (F) and reverse (R) strands.

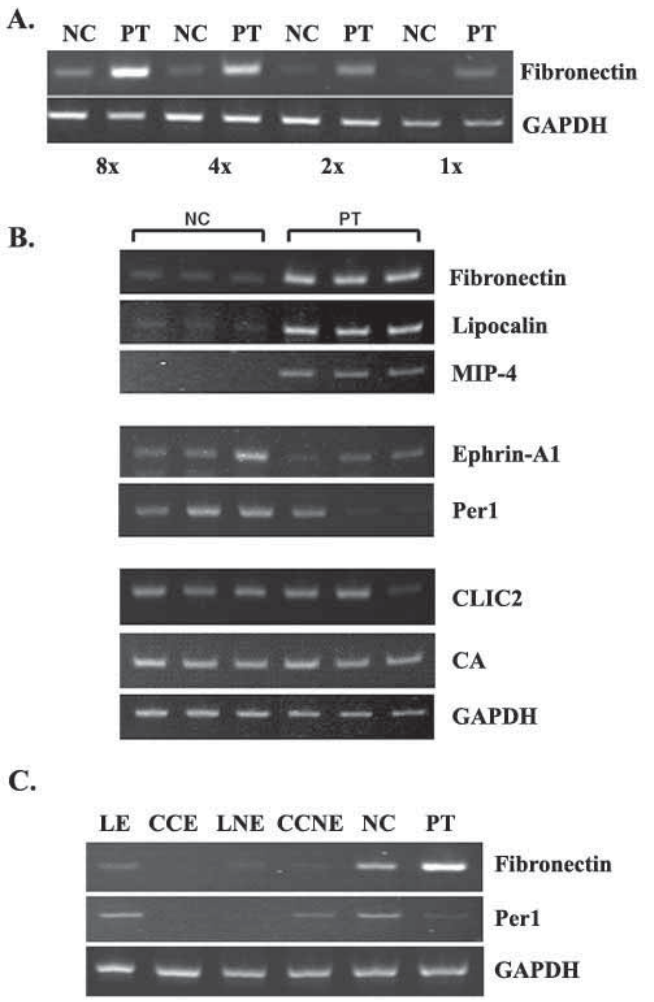

Figure 1. RT-PCR of normal conjunctiva and pterygium. **A**: Serial dilutions and densitometric imaging were used to compare the mRNA levels in primary pterygium (PT) and normal conjunctiva (NC). **B**: Higher mRNA levels of fibronectin, lipocalin, and macrophage inflammatory protein (MIP-4) and lower levels of ephrin-A1 and Per1 were observed in PT compared to NC. No significant difference was observed for chloride intracellular channel 2 (CliC2), carbonic anhydrase (CA), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **C**: Comparison with additional tissues showed that fibronectin mRNA levels were highest in PT compared to limbal epithelium (LE), central corneal epithelium (CCE), limbal nonepithelium (LNE), central corneal nonepithelium (CCNE), and normal conjunctiva (NC). Per1 mRNA levels were lower in PT than in LE, CCNE, and NC. Per1 mRNA levels were below the level of detection in CCE and LNE.

and extracellular matrix proteins comprised of fibronectin, collagen type III, versican, extracellular matrix protein 1, von Willebrand factor, collagen type VI, CD31 antigen, and cartilage oligomeric matrix protein.

Confirmation by RT-PCR: Gene expression in three primary whole pterygia and the autologous conjunctiva were evaluated by RT-PCR. As with the microarray data, fibronectin, lipocalin, and MIP-4 gene transcripts were more abundant and ephrin-A1 and Per1 were less abundant in pterygia compared to conjunctiva (Figure 1B, Table 4). The levels of expression for these genes were similar with the two methods (Table 4) with the exception of MIP-4, which potentially could be far greater than 5 fold. Some variability among samples was observed for Per1 and CLIC2. Statistical analysis indicated that Per1 gene transcripts were significantly decreased in pterygium compared to conjunctiva, while CLIC2 gene transcript levels were not significantly different between the two tissues. In contrast to the microarray data, RT-PCR showed no difference in carbonic anhydrase gene expression between pterygium and normal conjunctiva.

UV-damaged limbal stem cells are thought to give rise to pterygia based on shared cytokeratin and vimentin characteristics, although this continues to be an area of active investigation. To expand our comparative study to include putative pterygium progenitor cells, we examined limbal epithelium and nonepithelial cells plus other areas of the cornea. Three pterygia and dissected tissues from three separate donor eyes were compared (Figure 1C). Fibronectin mRNA levels were higher in pterygium compared to limbal epithelium, central cornea epithelium, limbal nonepithelium, central cornea

Table 2. Microarray analysis of human pterygium and normal autologous conjunctiva

Affymetric name	Genbank accession number	Fold change	Name	Description
Increased expression in pterygium				
311_s_at	X02761	8.5	Fibronectin	cell adhesion and migration
34290_f_at	AA995126	5.0	TRAP100	thyroid hormone receptor-associated protein
32128_at	Y13710	4.9	MIP-4	chemokine; pro-inflammatory
32488_at	X14420	4.1	collagen III	cell adhesion and migration
34778_at	AA418080	3.8	leucine rich repeat	integral to membrane
38634_at	M11433	3.6	RBP1	ligand trafficking; muscle malignancy
38111_at	X15998	3.3	versican, V1	matrix expansion in arterial and smooth muscle cells
37600_at	U68186	3.2	ECM 1	extracellular matrix protein 1
34015_at	AF004021	3.2	prostaglandin receptor	role in smooth muscle contraction
32582_at	AF001548	3.1	myosin heavy chain 11	smooth muscle component
40500_at	AF007138	3.1	NDRG4	response to stress and cell differentiation
34665_g_at	X62573	2.9	receptor for CD32	signal transduction, cell growth and maintenance
38351_at	AL050154	2.8	mRNA; clone	from clone DKFZp586L0120
37629_at	M55268	2.5	casein kinase 2	phosphorylation and signal transduction
39710_at	U30521	2.5	C5orf13	chromosome 5 open reading frame 13
607_s_at	M10321	2.5	von Willebrand factor	platelet/cell adhesion, binds to collagen III
32523_at	M20470	2.4	clathrin	protein binding; membrane trafficking
31610_at	U21049	2.4	MAP 17	extracellular integral to membrane
32821_at	A1762213	2.4	lipocalin 2	oncogene 24p3; transporter activity
41362_at	X91249	2.3	ABCG1	cholesterol and phospholipid transport
34757_at	AA595596	2.3	ADPRTL2	ribosyltransferase activity
38327_at	M77348	2.2	SILV	maturation of melanosomes
41471_at	W72424	2.2	calgranulin B	inflammatory response; cell-cell signaling
37269_at	D38496	2.2	LZTR1	leucine-zipper-like transcriptional regulator 1
479_at	U53446	2.1	DAB2	cell proliferation, signal transduction
38077_at	X52022	2.1	collagen type VI	cell adhesion and muscle development
37398_at	AA100961	2.1	CD31 antigen (PECAM1)	endothelial cell adhesion, signal transduction
33371_s_at	NM_006868	2.0	RAB31	member of RAS oncogene family
40161_at	L32137	2.0	COMP	cartilage oligomeric matrix protein
Decreased expression in pterygium				
40013_at	BC022305	-2.0	CLIC2	chloride intracellular channel 2
36829_at	AF022991	-2.0	Per1	circadian pacemaker protein
40425_at	NM004428	-2.0	ephrin-A1	ligand for EPH group of receptor tyrosine kinases
33373_at	AL049951	-2.0	FUBP	binds RNA; may function in control of splicing
37624_at	M29458	-2.0	carbonic anhydrase	family members are highly tissue-specific

The table lists the Affymetric probe set name, the Genbank accession number, the fold change in gene expression in human pterygium relative to normal autologous conjunctiva by microarray analysis, and a brief description of the name or major function of the gene product. The genes shown are statistically significant with $p<0.05$.

nonepithelium, and normal conjunctiva. Per1 mRNA levels were lower in pterygium compared to limbal epithelium, central cornea nonepithelium, and normal conjunctiva. Per1 mRNA was not detected in central cornea epithelium or in limbal nonepithelium.

Western blot analysis: Antibodies to fibronectin and MIP-4 were used to examine the level of protein expression in primary whole pterygium, cornea, and limbus. Western blot analysis showed that fibronectin (Figure 2B) and MIP-4 (Figure 2C) were increased in pterygium samples compared to limbal epithelium, central cornea epithelium, limbal nonepithelium, central cornea nonepithelium, and normal conjunctiva (Figure 2). The protein expression profiles for fibronectin and MIP-4 were consistent with the RT-PCR results (Figure 1).

Immunohistochemistry: Immunohistochemical analysis showed specific, increased staining of fibronectin, lipocalin 2, and MIP-4 in primary pterygium relative to cornea (Figure 3). Abundant fibronectin staining was noted along the entire length of the pterygium stroma and in some stromal fibroblasts (Figure 3B). Lipocalin 2 immunoreactivity was localized most intensely to basal epithelial cells, basement membrane, and to some stromal extracellular matrix (Figure 3D). Staining was also observed in normal corneal basal epithelial cells (Figure 3C). MIP-4 was widely distributed in the stroma and the overlying epithelial cell layers with expression in both the mature and immature epithelial cells (Figure 3F). Since the existence of lipocalin-type prostaglandin D synthase (L-PGDS) in a number of ocular tissues and in some tumors has previously been reported, it was included in the imunohistochemical study. The protein was differentially localized to basal epithelial cells (Figure 3H).

Table 3. Differentially expressed, pterygium-associated genes assembled into functional categories

Gene	Fold increase
Cell adhesion/extracellular-matrix-related	
Fibronectin	9.0
Collagen type III	4.1
Versican	3.3
Extracellular matrix protein	
Von Willebrand factor	2.5
Collagen type VI	2.1
CD31 antigen	2.1
Cartilage oligomeric matrix protein	2.0
Oncogenes	
Lipocalin 2 (oncogene 24p3)	2.4
RAB 31	2.0
Inflammatory response	
MIP-4	4.9
Calgranulin B	2.2
Smooth muscle contraction	
Prostaglandin Receptor F (2-alpha)	3.2
Myosin heavy chain 11	3.1
Signal transduction	
CD32 (IgG Fc Receptor)	2.9
Casein kinase II	2.5
DAB2	2.1

Gene names and the fold increase in gene expression observed in pterygium compared to normal autologous conjunctiva. Differentially expressed, pterygium-associated genes are categorized according to similarity in function.

DISCUSSION

These results have led to the identification of a set of differentially expressed genes in pterygium that may be important in the pathogenesis of this disease. Significant, and in several cases, substantial differences in expression were observed between whole pterygium (which is comprised of epithelial cells and fibroblasts) and normal conjunctiva, and other corneal and limbal regions. The list of genes (Table 2) provides novel targets for future investigations.

Our study confirms that pterygium is associated with oncogenic, angiogenic, fibrogenic, and inflammatory factors and, further, provides evidence of underrepresented genes that may contribute to pathogenesis.

A number of cell-adhesion and extracellular-matrix-related genes were increased in pterygium, including fibronectin, versican, extracellular matrix protein 1, cartilage oligomeric matrix protein, and collagen III and VI. The presence of fibronectin and collagen III is consistent with the immunohistochemical findings of Naib-Majani et al. [28]. Also, there is fair agreement on fibronectin localization. We found strong antibody staining of fibronectin in the stroma, as did Naib-Majani et al. [28], although they also reported staining in the basal epithelial cells.

Damage to the cornea by debridement or incision leads to a rapid accumulation of fibronectin. This adhesive glycoprotein functions as a temporary matrix for corneal epithelial

Table 4. Comparison of expression analysis results between microarray and semiquantitative RT-PCR

Gene	Microarray	RT-PCR
Fibronectin	8.5	8.4
Lipocalin	2.4	6.2
MIP-4	4.9	only in pterygium
Per1	-2.0	-1.6
Ephrin-A1	-2.0	-1.6
Carbonic anhydrase	-2.0	1.1
CliC2	-2.0	1.2
GAPDH	1.0	1.0

The gene name and fold change for pterygium compared to conjunctiva are shown for microarray analysis and semiquantitative RT-PCR. Genes with high differential expression by microarray were selected for comparison. GAPDH was used as a control.

cell attachment and migration [29-32]. After corneal resurfacing, fibronectin disappears. It seems plausible that the accumulation of fibronectin in the fibrovascular stroma of the pterygium serves the same function, providing a substratum for pterygial cell migration.

Fibronectin has also been associated with malignant transformation and resistance to apoptosis of tumors and may play a similar role in pterygium. Early signs of malignant transformation include the production of fibronectin by the peritumoral stroma [33]. Accumulation of fibronectin has been noted in a number of malignancies including breast, lung, and pancreatic cancers [34-36]. Han et al. [35] showed that addition of fibronectin to cultured human lung carcinoma cells increased proliferation and substantially decreased apoptosis, reflected by the reduced amount of DNA fragmentation in cells cultured in serum-free medium. Pterygium is considered a benign neoplastic lesion with characteristics of transformed cells including loss of heterozygosity and microsatellite instability [26,37,38]. In addition, pterygium displays reduced apoptosis with increased levels of the apoptosis-inhibiting protein bcl-2 [16]. It is possible that the increased level of fibronectin in pterygium facilitates cell proliferation with reduced apoptosis.

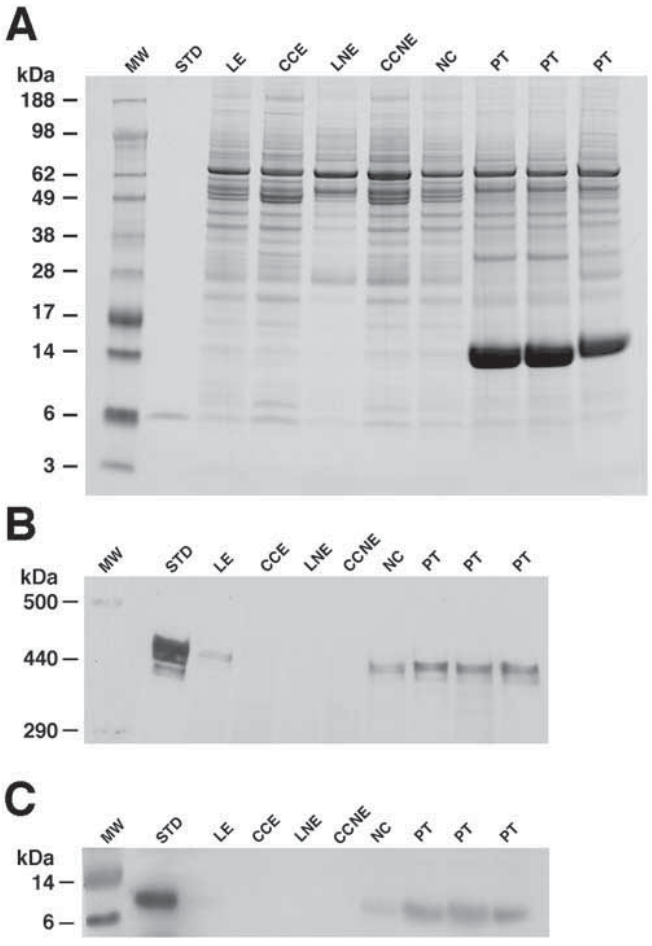

Figure 2. Comparison of protein levels in pterygium to other tissues by western blot. **A**: Representative protein loading on SDS-PAGE (3-188 kDa) gels. Western blots show that the relative levels of fibronectin (**B**) and MIP-4 (**C**) are increased in pterygium (PT) compared to limbal epithelium (LE), central cornea epithelium (CCE), limbal nonepithelium (LNE), central cornea nonepithelium (CCNE), and normal conjunctiva (NC). Molecular weight standards (MW) and fibronectin and MIP-4 purified proteins (STD) were used to verify the relative mobility of the two proteins.

Lipocalins are extracellular proteins that bind and transport a variety of low-molecular-weight molecules, including iron and lipophilic ligands such as retinoids, steroids, pheromones, and odorants [39-41]. Because of the diversity of ligands transported, lipocalins can have an impact upon a wide range of cellular functions. Lipocalins may play a biophysical role in some tissues. For example, it has been suggested that human tear lipocalin (TL), which accounts for about 15-33% of the tear proteins, increases the solubility of lipids in the tear film and reduces evaporation of water from the corneal surface [42-45]. Some lipocalins also serve as biochemical markers of inflammatory diseases, cancer, and lipid disorders [46].

Our microarray data indicates that retinol-binding protein 1 and lipocalin 2 are associated with pterygium. Another lipocalin family member, L-PGDS, a PGD_2-producing enzyme and retinoid transporter, has previously been reported in a number of ocular tissues and in some tumors [47-51]. L-PGDS represents the most abundant species in not only an unnormalized NEIBank mouse rpe/choroid cDNA library (NbLib0059) but in a human corneal endothelial cDNA library as well [52]. We therefore examined the immunohistochemical distribution of this lipocalin and found it to be differentially localized to pterygial basal epithelial cells.

Immunostaining of lipocalin 2 (oncogene 24p3) was increased in pterygium and localized to the basal pterygial epithelial cells, basement membrane, and some areas of the extracellular stroma. It is known to be highly expressed in colonic epithelium in areas of inflammation, both in nonmalignant epithelium (diverticulitis, inflammatory bowel disease, and appendicitis) and in premalignant and malignant neoplastic lesions of the colon [53]. It is also associated with hepatic, pancreatic, breast, and ovarian cancers [54-57].

Lipocalin 2 is also known as neutrophil gelatinase-associated lipocalin (NGAL) [58,59], due to its binding and activation of latent matrix metalloproteinases, in particular MMP-8 and MMP-9 [39,56,59,60]. As such, it has been proposed to play a role in tissue remodeling and metastasis. It is noteworthy that NGAL is highly expressed in the pterygium basal epithelial cells, considering that we previously observed strong immunostaining of a number of matrix metalloproteinases, including MMP-9, in the same cellular location [21]. The colocalization suggests that NGAL may augment the MMP-driven dissolution of Bowman's layer and invasion by limbal basal epithelial cells.

The chemokine MIP-4, also called pulmonary and activation-regulated chemokine (PARC), dendritic cell derived C-C chemokine (DCCK) 1, and alternative macrophage activation associated C-C chemokine (AMAC) [61], was highly expressed in pterygium, providing further evidence of the proinflammatory nature of the lesion [1]. MIP-4 functions as a chemoattractant for $CD4^+$ and $CD8^+$ T lymphocyte subpopu-

lations (indicating humoral and cell-mediated regulation), with no effect on activated T lymphocytes, granulocytes, or monocytes [61]. It has been reported in a number of tissues, including alveolar macrophages and dendritic cells, which are potent antigen presenting cells [62,63]. Langerhans' cells, which belong to the dendritic mononuclear cell family, and macrophages have been identified in pterygium [64] and could be the source of secreted MIP-4. The strong staining of this protein in the fairly homogeneous epithelial layer, however, seems to suggest that these cells may also secrete MIP-4.

Blood vessels are a major component of the pterygium stroma. Our microarray data indicated a 2 fold increase in CD31 antigen, which is involved in intercellular adhesion of endothelial cells [65,66]. We also observed a 2.5 fold increase in von Willebrand factor in pterygium compared to conjunctiva. Our findings are in general agreement with Naib-Majani et al. [28], who detected this protein in pterygium blood vessel walls. von Willebrand factor is essential for platelet adhesion, and it binds directly to collagen III [67]. Collagen III is also overexpressed in pterygium.

Disruption of circadian rhythm has been associated with malignancy. Per1 is a core component of the circadian system and was underexpressed in pterygium compared to conjunctiva, limbal epithelium, and central cornea nonepithelium. Expression of Per1 is significantly decreased in endometrial carcinoma and in breast cancer [68,69]. Matsuo et al. [70]

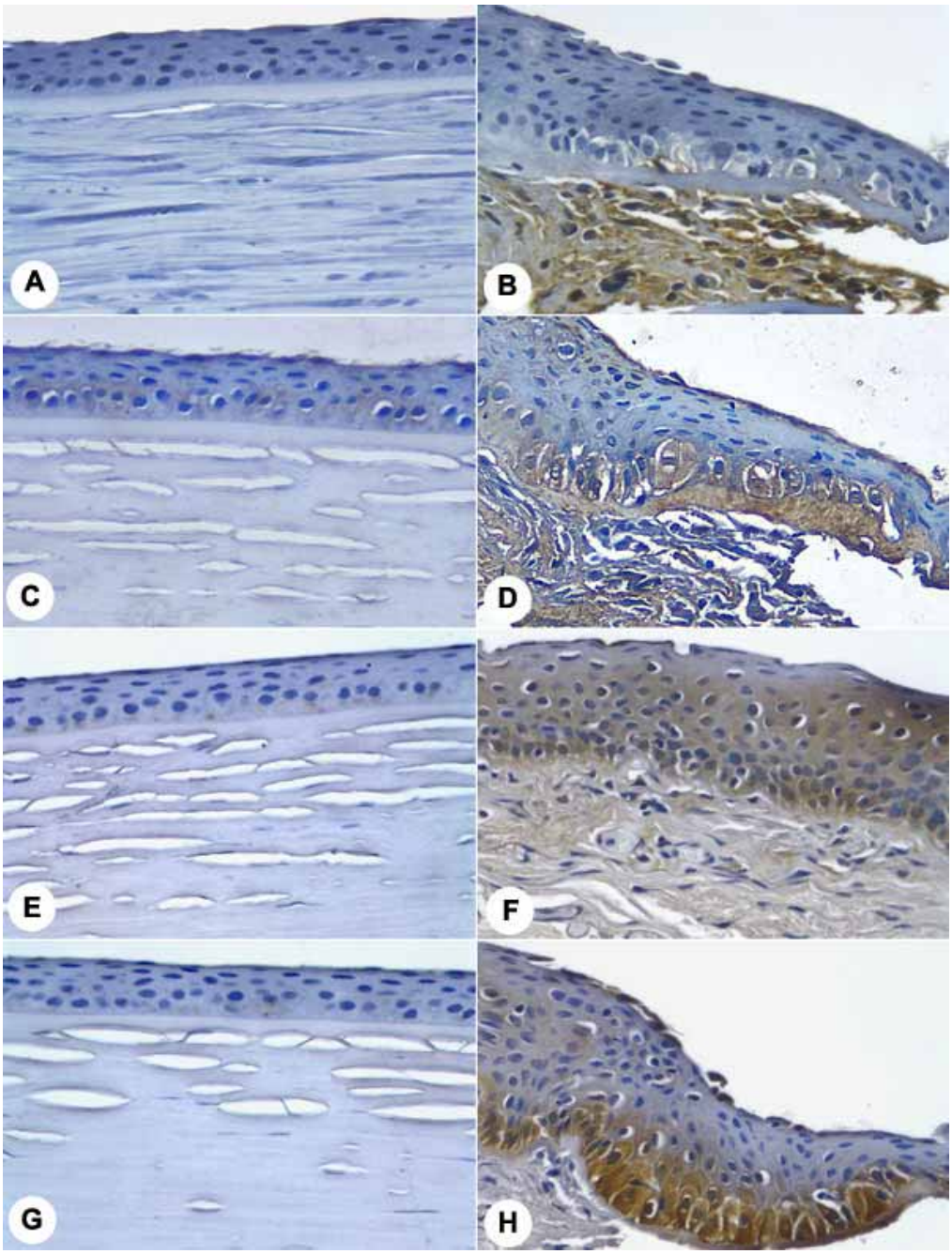

Figure 3. Immunohistochemical analysis of proteins in pterygium and normal cornea. Immunohistochemical staining for fibronectin (**A**,**B**), lipocalin 2 (**C**,**D**), MIP-4 (**E**,**F**), and L-PGDS (**G**,**H**) was increased in pterygium (right panels) when compared to normal cornea (left panels). **B**: Abundant fibronectin staining occurs along the entire length of the pterygium stroma and in some stromal fibroblasts. **C**: Lipocalin 2 is faintly detected in normal corneal basal epithelial cells. **D**: Lipocalin 2 immunoreactivity is localized to pterygial basal epithelial cells, basement membrane, and some extracellular components of the stroma. **F**: MIP-4 is present in both the stroma and the epithelial cell layers. **H**: Intense staining of L-PGDS was observed in the basal epithelial cell layer. A thinly stained line at the pterygial and corneal cell surface may either be a specific reaction by terminally differentiated epithelial cells or a possible immunological edge effect. Protein localization in the specified areas was the same in the wider field seen under lower magnification.

showed that the circadian clock controls the expression of cell cycle-related genes, and it has been suggested that the decrease in Per1 deregulates the cell cycle, favoring proliferation [68-70].

Our microarray data confirmed a number of genes that had previously been reported in pterygium. In addition, this global gene expression approach identified many new genes that were either over- or underrepresented in pterygium. In particular, several cell adhesion/extracellular matrix proteins and proteins involved in inflammation and malignant transformation had increased expression levels. The finding that lipocalin 2 (NGAL) is overexpressed in pterygium is consistent with the functional activation of MMPs by NGAL. MMPs are matrix-degrading enzymes that have been implicated in the pathogenesis of pterygium. Inhibition or silencing of NGAL might inactivate this pathway and the progression of pterygium. Other areas that could be addressed include further evaluation of these genes in recurrent pterygium and identification of genes that are singularly representative of primary or recurrent pterygium or of selected morphological areas such as the epithelial cell leading edge.

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