

Analysis of Gene Expression in Human Bullous Keratopathy Corneas Containing Limiting Amounts of RNA

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PURPOSE. To validate the use of polymerase chain reaction (PCR)-amplified full-length cDNA as a substitute for mRNA in nucleic acid array and gene expression analysis.

METHODS. Total RNA was isolated from age-matched normal autopsy corneas and pseudophakic bullous keratopathy (PBK) corneas. Full-length cDNA was generated and PCR amplified using the Smart cDNA synthesis technology. Southern blot analysis of this cDNA was compared with Northern blot analysis of the RNA. Amplified cDNA was used to probe a commercial gene array. By immunohistochemistry, the expression pattern of several adhesion molecules represented on the array was assessed.

RESULTS. The cDNA produced by the Smart cDNA system gave results very similar to those of northern blot analysis when examined for β_2 -microglobulin, Rab geranylgeranyl transferase, and tenascin-C. This cDNA obtained from normal or PBK corneas was labeled and used to probe a 588 gene array (Clontech). Among other differences, β_6 integrin was detected only with the PBK probe, β -catenin was markedly elevated in PBK, and β_4 integrin appeared to be reduced in PBK. Immunohistochemical patterns of these proteins were consistent with the hybridization signals on the gene array.

CONCLUSIONS. Smart cDNA synthesis and nucleic acid arrays were combined and validated for the first time to identify differential gene expression in normal and diseased corneas. These techniques require very little RNA such as that equivalent to a half of a single cornea, which is useful when the amount of tissue is limiting. Altered expression of adhesive proteins β_6 integrin and β -catenin may be related to the formation of epithelial bullae and microcystic changes in PBK patients. (*Invest Ophthalmol Vis Sci.* 1999;40:3108-3115)

Recently, large-scale nucleic acid arrays have become commercially available. These arrays can be extremely useful to investigators exploring differences in gene expression between cell types, stages of differentiation, or cellular responses to stimuli and changes in environment. For example, Clontech (Palo Alto, CA) supplies high density arrays composed of approximately 600 transcriptionally regulated cDNAs arranged into functional classes that also include non-regulated housekeeping genes (Atlas Human cDNA Array).

Reverse transcription of poly(A)⁺ RNA in the presence of α -³²P dATP or dCTP, and utilization of the resulting labeled cDNA as a hybridization probe, allows one to quickly assess the relative expression level of each gene represented in the array in a single experiment. Furthermore, comparison of the hybridization patterns of two sources of RNA can identify differences in gene expression between these sources.¹ Clearly, this technology can have tremendous advantages in both cost and time over standard techniques of northern blot analysis, RNase protection assay, and reverse transcription-polymerase chain reaction (RT-PCR).

One drawback to this technique is the need to isolate and purify microgram amounts of poly(A)⁺ RNA to generate the appropriate probe. In studies concerning human corneal diseases, the amount of available tissue is very limited and often represents only a portion of a cornea. Typically, these surgical samples contain 2 to 5 μ g of total RNA, well below the amounts needed. One option to circumvent this problem is the use of primary cell cultures. However, the results may not be comparable to those obtained with the intact cornea.^{2,3}

Smart cDNA synthesis technology has been recently introduced by Clontech. It allows one to generate full-length cDNA from the RNA pool, PCR amplify it, and then use the amplified cDNA as a hybridization probe. This method is supposed to generate cDNA representative of the mRNA pool; for this reason, Southern blot analysis using this cDNA has been called

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“virtual Northern” analysis.^{4,5} However, to the best of our knowledge, virtual Northern has not been compared so far with conventional Northern in terms of size and representation of individual mRNAs despite the importance of such validation. To fill this gap, we have validated the protocol using conventional Northern blot analysis of RNA from human normal or pseudophakic bullous keratopathy (PBK) corneas. The results showed that Smart cDNA synthesis technology generated adequate information concerning mRNA size and relative gene expression in small tissue samples. We were able to reproducibly use virtual Northern with starting amounts of RNA equivalent to one half of a cornea, as opposed to conventional Northern blot analysis that required pooled RNA from 20 corneas. The cDNA generated by this method was further used as a probe to screen gene arrays for differences in gene expression between normal and PBK corneas.

PBK is a corneal disorder that occurs after cataract removal with placement of an artificial intraocular lens. If there has been no lens placement, the ensuing disorder is referred to as aphakic bullous keratopathy, which is clinically and histologically indistinguishable from PBK. The present therapy for PBK is treatment with topical corticosteroids and hypertonic solutions. However, despite therapeutic efforts, PBK continues to be a leading indication for corneal transplantation.⁶ The clinical hallmarks of this disease are chronic corneal edema, possibly due to corneal endothelial cell dysfunction, subepithelial bullae (blisters), and eventual loss of transparency.⁷⁻¹⁰ It is also characterized by extensive fibrosis, with abnormal deposition of extracellular matrix proteins tenascin-C and fibrillin-1.^{11,12} In advanced fibrotic cases, a posterior collagenous layer, or retrocorneal fibrous membrane, is formed between corneal endothelium and Descemet's membrane.¹⁰ In this study of gene expression using nucleic acid array we were particularly interested in select cell-substratum and cell-cell adhesion molecules because PBK corneas have frequent detachments of the epithelium and subepithelial fluid-filled bullae. Therefore, we focused on the integrin and catenin patterns present on nucleic acid arrays. Many other differences that were observed were not pursued further at this time with validation of gene array data at the protein level.

Integrins are dimeric molecules composed of an α and a β subunit that allow the cell to attach and respond to various extracellular matrix proteins (e.g., fibronectin, collagens, tenascin, and laminins).¹³ Integrins are important in cell adhesion, migration, and differentiation.¹⁴⁻¹⁶ Some integrins are constitutively expressed in the cornea,^{17,18} whereas others change in the wound-healing process or in disease states.¹⁹⁻²¹ Catenins are cytoplasmic proteins that form complexes with cadherins and mediate cell-cell adhesion. There are 3 catenins, α -, β -, and γ - (the latter of which is also known as plakoglobin), that are associated with cytoskeletal actin filaments.²²⁻²⁵ Catenins have been found in rabbit corneal fibroblasts and myofibroblasts²⁶ and in mouse corneal epithelial cells.²⁷ The presence of catenins within human normal or diseased corneas has not been examined. The data presented here show that by gene array analysis and immunohistochemistry PBK corneas had increased expression of β -catenin and β_6 integrin. These alterations may be related to epithelial adhesion abnormalities in diseased corneas.

METHODS

Tissue and RNA Isolation

Normal corneas were received within 24 hours after death from the National Disease Research Interchange (Philadelphia, PA). Age-matched PBK corneas and keratoconus corneas used as controls were received within 24 hours of penetrating keratoplasty from a network of collaborating surgeons. Because only the central corneal buttons (~8 mm in diameter) were available for PBK corneas, normal corneas were trephined, and only the central portions were used. For surgical specimens of PBK and keratoconus corneas, only one half of each corneal button was often available for RNA analysis because the other half was sent for pathologic examination. Corneas were snap-frozen on arrival and stored in liquid nitrogen until use. For RNA analyses, they were pulverized to a fine powder under liquid nitrogen with the use of a mortar and pestle. One milliliter of Trizol (Life Technologies, Gaithersburg, MD) was added to the frozen powder and allowed to warm to room temperature. Chloroform (0.2 ml) was added per 1 ml Trizol, and the mixtures were incubated for a further 3 minutes. The phases were separated by centrifugation at 12,000g for 15 minutes at 4°C. The resultant aqueous phase was removed, 0.5 ml isopropanol was added to precipitate the RNA, and the samples were again centrifuged at 12,000g for 15 minutes at 4°C. The pellets were washed with 75% ethanol, dried under vacuum, and resuspended in deionized water. The yield and purity of RNA were estimated by optical density at 260/280 nm.

Northern Blot Analysis

Northern blot analysis was carried out by a standard method. Briefly, 20 μ g of isolated RNA from pooled normal ($n = 20$) or PBK corneas ($n = 20$) was separated on 1.2% denaturing agarose gels, containing 2.2 M formaldehyde. RNA was then transferred by capillary action to Hybond N⁺ (Amersham, Arlington Heights, IL). After transfer, RNA was cross-linked to the membrane with the use of a Stratalink (Stratagene, San Diego, CA). The probes were generated by random priming the cDNAs of interest. Hybridizations were conducted at 42°C in 50% formamide, 5× SSC (1× = 150 mM NaCl, 15 mM sodium citrate), pH 7.0, 1× Denhardt's solution (5 Prime → 3 Prime, Inc., Boulder, CO), 10 mM sodium phosphate, pH 6.8, 0.01% sodium dodecyl sulfate (SDS), 2.5 mM EDTA, 5 μ g/ml poly(A)_n, 50 μ g/ml salmon sperm DNA, and 50 μ g/ml yeast RNA. Washings were at 50°C with 0.1% SSC, pH 7.0, and 0.01% SDS. The hybridized probes were visualized by autoradiography.

cDNA Synthesis

Equivalent amounts of RNA from either five normal or five PBK full central corneal buttons were mixed to generate pools of normal or diseased corneal RNA. Pooling was done to minimize the variations within the population group. One microgram of the pooled RNA from each source (equivalent to one half of a single cornea) was reverse-transcribed using the protocols and reagents supplied in the Smart cDNA Synthesis kit (Clontech). Briefly, RNA was mixed with a modified oligo(dT) primer (final concentration = 2 μ M), and an additional oligonucleotide (AAGCAGTGGTATCAACGCAGAGTACGCGGG, final concentration = 1 μ M), in a volume of 5 μ l. The mixture was incubated at 70°C for 2 minutes and then immediately chilled

on ice for a further 2 minutes. Five microliters of a master mixture composed of 50 mM Tris-HCl (pH 8.3), 6 mM MgCl₂, 75 mM KCl, 1 mM dNTPs, 2 mM dithiothreitol and 200 U SuperScript reverse transcriptase (Life Technologies) was added. The reaction was allowed to proceed for 1 hour at 42°C and then terminated by heating to 72°C for 10 minutes. This procedure relies on the fact that when SuperScript reaches the 5' end of the template, its terminal transferase activity adds additional nucleotides to the 3' end of the cDNA. The additional primer included in the reaction hybridizes with the terminal tail, and the transcriptase switches strands and incorporates the primer sequence into the cDNA. According to Clontech, this protocol will generate full-length cDNAs representative of the input mRNA and containing defined sequences at both the 3' and 5' ends.

cDNA Amplification and Probe Generation

The cDNA obtained was diluted 1:5, 1:25, or 1:125 with 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and stored at -20°C until use. One microliter of diluted cDNA was then mixed with a master mix composed of 40 mM Tricine-KOH (pH 9.2), 15 mM KOAc, 3.5 mM Mg(OAc)₂, 75 µg/ml bovine serum albumin, 0.2 mM dNTP, 0.2 µM AAGCAGTGGTATCAACGCAGAGT, and 1× Advantage KlenTaq Polymerase mix (Clontech). The cDNA was then PCR amplified using the following program profile: 1 cycle, 95°C, 1 minute; followed by 20 cycles of 95°C, 20 seconds; 68°C, 6 minutes; followed by 1 cycle, 72°C, 10 minutes.

Aliquots of the isolated PCR products were electrophoresed on 0.8% agarose gels containing Tris-acetate-EDTA (TAE) buffer and then transferred to Hybond N⁺ (Amersham) membranes using an alkaline transfer protocol. The membranes were then probed with a variety of probes labeled by random primer method.

To generate a probe for the nucleic acid array, the PCR products obtained after 20 cycles of amplification from either normal corneal RNA or PBK RNA were fractionated on ChromaSpin 1000 columns (Clontech) to remove unincorporated primers and small (<300 bp) products. The DNA concentration was then quantitated by ethidium bromide fluorescence. One hundred nanograms of each DNA sample was then labeled with α-³²P dCTP (3000 Ci/mmol) to specific activity of 10⁹ cpm/µg DNA using a random prime labeling kit (Life Technologies). After the reaction was stopped with EDTA, the unincorporated nucleotides were removed using Bio-Spin 30 columns (Bio-Rad Laboratories, Hercules, CA), and labeled probes were hybridized to a nucleic acid array (Atlas Human cDNA Expression Array, Clontech) in ExpressHyb solution (Clontech). After hybridization at 68°C overnight, the membranes were washed twice with 2× SSPE (1× = 150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4), 0.2% SDS at room temperature, followed by two washes at 68°C with 0.1× SSPE, 0.2% SDS. After washing, the membranes were analyzed with a phosphorimaging device (Storm; Molecular Dynamics, Sunnyvale, CA). The signal intensities for individual genes were normalized to the signals for several housekeeping genes.

Immunofluorescence

Indirect immunofluorescence on unfixed or 0.4% formaldehyde-fixed corneal cryostat sections was performed as described.²⁸ A total of 15 normal and 22 age-matched PBK cor-

neas were used. To verify that the changes seen were not related to postmortem changes or postsurgical storage in transportation media, tissue sections were also analyzed from two postmortem radial keratotomy corneas and 11 postsurgical keratoconus corneas. These were treated in a manner identical to that of the normal and PBK corneal tissue sections. In some experiments, to reveal a fraction of catenins associated with the cytoskeleton, unfixed corneal sections were treated for 5 minutes at room temperature with a modified²⁷ cytoskeleton buffer (CSK buffer) containing 50 mM NaCl, 10 mM PIPES, pH 6.8, 3 mM MgCl₂, 0.5% Triton X-100 and 30 mM sucrose.

Monoclonal antibodies were used to α-catenin (clone αCAT-7A4; Zymed Laboratories, San Francisco, CA), β-catenin (clone 14; Transduction Laboratories, Lexington, KY; and clone 15B8, Sigma Chemical, St. Louis, MO), γ-catenin (clone 15; Transduction Laboratories), E-cadherin (clone HECD-1; Zymed Laboratories), β₆ integrin (clone E7P6²⁹), β₄ integrin (clone 3E1; Chemicon International, Temecula, CA), and α₆ integrin (clone NKI-GoH3; Chemicon International), all at 10 to 20 µg/ml. Routine specificity controls without the primary or the secondary antibodies were routinely included and were negative. Cross-species adsorbed secondary antibodies were from Chemicon International.

RESULTS

Validation of the Smart cDNA Synthesis by Northern Blot Analysis

Nucleic acid arrays can be a powerful way to assess the relative gene expression of known transcripts in a rapid manner. However, the amount of poly(A)⁺ RNA required to generate a cDNA probe to be used with an array cannot always be obtained from small tissue samples. The possibility has been previously considered that PCR-amplified cDNA obtained from the Smart cDNA synthesis reaction may be used as an alternative to poly(A)⁺ RNA.⁴ To be useful for this purpose, the amplification products must reflect the diversity, size, and representation of the input mRNA pool. To assess this aspect of the amplification protocol, the products from the Smart cDNA synthesis reaction were examined for specific cDNA products. Total RNA from pooled normal and PBK samples was reverse-transcribed, diluted, and then amplified for 20 cycles. The resultant cDNA products were then assessed by Southern blot analysis (virtual Northern) for the presence of appropriately sized cDNA products known to be present in the input mRNA pool. In addition, the relative proportion of the various products obtained after amplification was compared with a Northern blot analysis of the initial RNA pool.

As expected, both the original RNA pool and the amplified cDNA contained material that hybridized with a probe for β₂-microglobulin, a housekeeping gene (β₂-microglobulin [β₂-MG], Fig. 1). On both blots, the probe hybridized to a single band of 1.6 kb, which is consistent with the known size of β₂-MG. However, the hybridization to the normal samples was lower than that observed for the PBK samples. To more precisely quantify the hybridization, the bands were counted using a Radioanalytical Imaging System (Ambis System, San Diego, CA). By Northern blot analysis, the amount of hybridization was approximately 6 times higher in the PBK sample than in the normal sample. By Southern blot analysis (virtual Northern), the amount of β₂-MG hybridization was 5.7 times

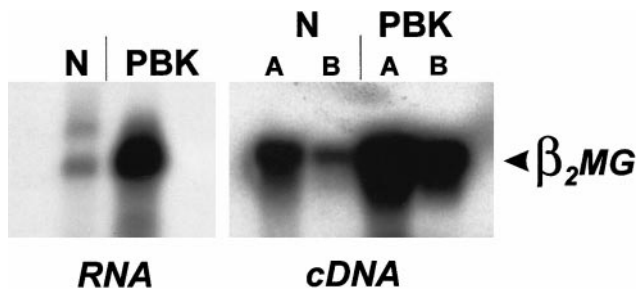


FIGURE 1. Northern blot analysis of normal and PBK RNA compared with Southern blot analysis of amplified cDNA. *Left:* 20 μ g of RNA was electrophoresed and transferred to membranes. *Right:* One microgram of either normal or PBK RNA was reverse transcribed and amplified as described in the Methods section. The cDNA was diluted either 1:25 (A) or 1:125 (B) and then amplified for 20 cycles, electrophoresed, and transferred to membranes. Both membranes were then probed for β_2 -MG. *Arrowhead* indicates the approximate position for the probe used. N, normal.

higher in PBK versus normal samples diluted either 1:5 (not shown) or 1:25 (Fig. 1, right panel, A lanes) and 3.6 times higher in the sample diluted 1:125 (Fig. 1, right panel, B lanes). This indicates that the absolute proportion of β_2 -MG was maintained in amplified cDNA samples for at least two dilutions (1:5 and 1:25), and the relative proportion was maintained regardless of the amplification parameters.

Although it appeared that at least one housekeeping gene (β_2 -MG) was faithfully represented in the amplified cDNA, it was not clear if less abundant transcripts would also be represented, in the proper proportion, and of the appropriate size. Rab geranylgeranyl transferase (RGGT) is a low abundance, average-sized (~2-kb) transcript that is expressed in both normal and PBK corneas. Using this cDNA as a probe, Northern and Southern blot analyses were performed. As seen in Figure 2, the relative intensities of RGGT were similar on both blots, with more intense hybridization in the normal sample. In addition, the product size in both samples was the same (~2kb). By densitometric analysis, the proportion of RGGT in the PBK RNA sample relative to the normal was approximately

0.4. In the amplified cDNA samples, the apparent value was 0.4 in the 1:25 diluted sample (Fig. 2, right panel, A lanes) and 0.2 in the 1:125 diluted sample. Similar to results for β_2 -MG, the relative proportion of RGGT was maintained under the PCR conditions used, and the absolute proportion was maintained at 1:25 dilution.

The same blots were then stripped and reprobed for tenascin-C, a very low abundance, large (6–8 kb) gene product that is present in PBK corneas and absent in normal corneas.^{11,30,31} Specific hybridization at approximately 6 kb (the reported size for the small tenascin-C mRNA variant common in PBK) was observed in the PBK RNA and its corresponding cDNA. No specific hybridization for tenascin-C was seen in the normal samples (Fig. 2). Taken together, these results for β_2 -MG, RGGT, and tenascin-C indicated that the amplified cDNA contained full-length cDNA products. Furthermore, the products were present in the appropriate proportions relative to the RNA as judged by Northern blot analysis and under certain conditions (the 1:5 or 1:25 dilutions), the absolute proportion of the products was maintained. This suggested that the amplified cDNA (using a 1:25 dilution of the RT reaction) could reliably be used as a probe on nucleic acid arrays as a substitute for the standard method that uses cDNA directly labeled during reverse transcription of poly(A)⁺ RNA.

Gene Array Analysis

After isolation of the amplified cDNA by column chromatography to remove nucleotides, primers, and small products, the cDNA was labeled using the random primer method. This material was then allowed to hybridize to an Atlas Human cDNA Expression Array and analyzed using the Storm Phosphorimager (Molecular Dynamics). Numerous differences between normal and PBK corneas were detected and are the subject of ongoing study. Here we have focused on the differences between normal and PBK corneas observed in the expression of adhesion molecules. These differences were verified at the protein level. In particular, it was noted that the PBK array contained good signals for β_6 integrin and β -catenin cDNA. In contrast, normal array (Fig. 3) was negative for β_6 integrin and only weakly positive for β -catenin (21 times less than PBK by

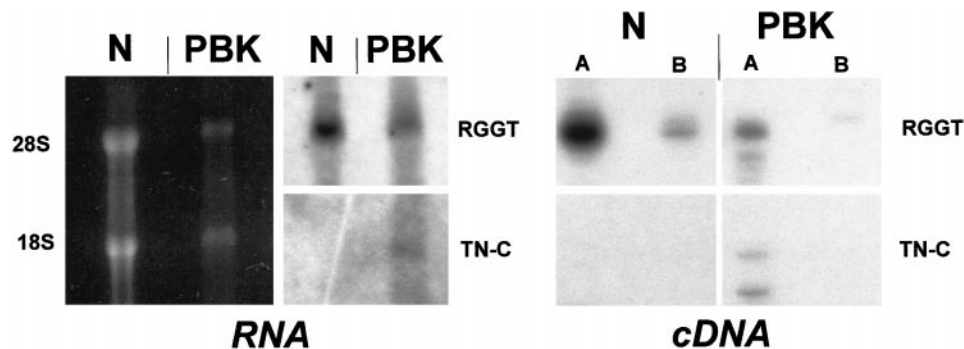


FIGURE 2. Northern blot analysis of normal and PBK RNA compared with Southern blot analysis of amplified cDNA for low level transcripts. *Left:* Twenty micrograms of RNA was electrophoresed and transferred to membranes. *Right:* One microgram of either normal or PBK RNA was reverse transcribed and amplified as described in the Methods section. The cDNA was diluted either 1:25 (A) or 1:125 (B) and then amplified for 20 cycles, electrophoresed, and transferred to membranes. Both membranes were then probed for Rab geranylgeranyl transferase (RGGT), stripped, and then reprobed for tenascin-C (TN-C). In both Northern blot analysis for the RNA and Southern blot analysis of the cDNA, normal samples had higher levels of RGGT and PBK samples had more TN-C. N, normal.

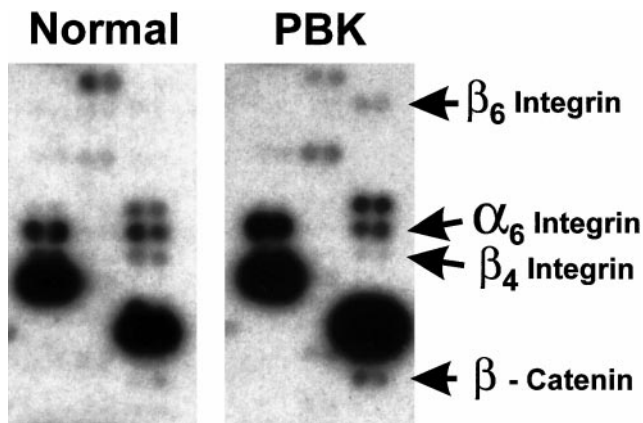


FIGURE 3. Nucleic acid array analysis demonstrating selected integrin and catenin gene expression. The normal and PBK cDNAs were generated as described in Figure 2, random prime labeled, and used to probe a commercial nucleic acid array. An area of the digitized image captured by a phosphorimaging device is shown. Arrows indicate the positions (2 spots for each) of the indicated gene products. PBK has higher expression of β_6 integrin and β -catenin, whereas normal has higher expression of β_4 integrin. α_6 integrin is comparable in normal and PBK corneas. All values are standardized to housekeeping genes present on the array (not shown).

phosphorimaging). The signal for β_4 integrin, on the other hand, was reduced on the PBK blot (3 times less than normal by phosphorimaging), whereas the signals from both the housekeeping cDNAs (not shown) and α_6 integrin appeared equivalent in the two blots.

Immunofluorescence Analysis of Integrin and Catenin Expression Patterns

To assess whether these apparent differences were reflected at the protein level, monoclonal antibodies specific for α -, β -, and γ -catenin; E-cadherin; and α_6 , β_4 , and β_6 integrin subunits were used to examine corneal sections by indirect immunofluorescence. As seen in Figure 4, immunostaining for α_6 integrin (Figs. 4A and 4B) was indistinguishable between normal (Fig. 4A) and PBK (Fig. 4B) corneas. The staining for β_4 integrin (Figs. 4C and 4D) showed that both normal (Fig. 4C) and PBK (Fig. 4D) corneas had a continuous band of fluorescence at the level of the epithelial basement membrane. However, in PBK corneas, this staining was less intense than that seen in normal corneas and occasionally discontinuous. These staining patterns were in agreement with the results of the nucleic acid array.

When the staining patterns for β_6 integrin (Figs. 5A through 5C) and β -catenin (Figs. 5D through 5F) were examined, marked differences were apparent between the normal (Figs. 5A and 5D) and PBK (Figs. 5B and 5E) corneas. Immunostaining for β_6 integrin was negative in normal corneas (Fig. 5A). However, in most PBK corneas (12/15, Fig. 5B), cell membranes in the basal epithelial layer were positively stained, again in accordance with gene array data. To assess whether the appearance of β_6 integrin staining was related to surgical specimens, corneas surgically removed from keratoconus patients were also examined. As in normal corneas, no specific staining was observed in the majority of keratoconus corneas (8/11 negative, Fig. 5C).

The antibodies to β -catenin specifically and strongly stained cell membranes in all epithelial cell layers in PBK

corneas (Fig. 5E), whereas significantly less staining (mostly in the superficial layers) was reproducibly seen in normal corneal sections (Fig. 5D). Corneal endothelium (not shown here) and keratocytes (Figs. 5D and 5E) were also positive, but their staining was similar in normal and PBK corneas. Extraction of corneal sections with CSK buffer to reveal the cytoskeleton-bound fraction of β -catenin²⁷ showed the same staining pattern as in respective untreated corneas (not shown here). To assess whether differential staining for β -catenin was related to surgical versus autopsy specimens, a postmortem radial keratotomy specimen was also examined. In this case, the antibody stained mostly the epithelial cells in plugs over the keratotomy scars (Fig. 5F). This indicated that weak β -catenin staining in normal versus PBK samples was not a postmortem artifact and that the increase of β -catenin might be related to trauma, fibrosis, or wounding of the tissue.

α -Catenin had a pattern largely similar to β -catenin and did not change appreciably in PBK. However, after CSK buffer extraction, much less α -catenin was seen in both normal and PBK epithelium, whereas the endothelial staining was unchanged (not shown here). γ -Catenin antibody strongly stained epithelial and endothelial cell membranes in both normal and PBK corneas (not shown here). E-cadherin antibody weakly stained epithelial and endothelial cell surfaces. In PBK corneas,

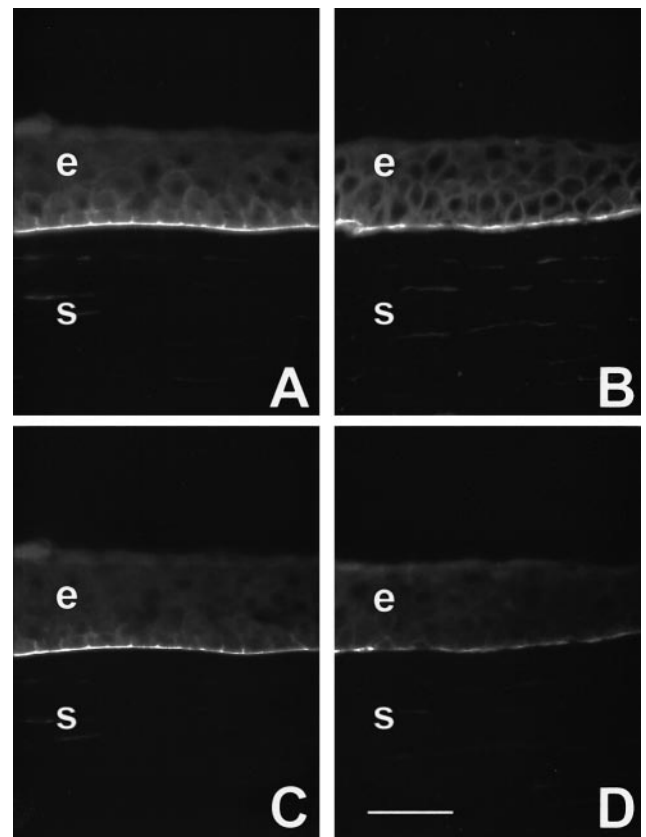


FIGURE 4. Immunohistochemical staining of normal and PBK corneas. Monoclonal antibodies to either α_6 (A and B) or β_4 (C and D) integrins were used to examine frozen sections of normal and PBK corneas by immunofluorescence. Normal corneas are shown in (A) and (C), PBK corneas are shown in (B) and (D). The staining patterns of α_6 integrin are comparable in normal and PBK corneas. β_4 integrin staining in PBK is discontinuous and weaker than that seen in normal corneas. e, epithelium; s, stroma. Scale bar, 40 μ m.

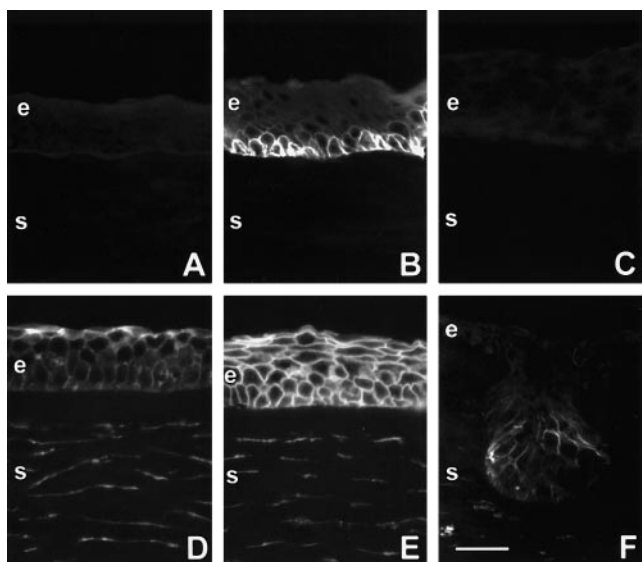


FIGURE 5. Immunohistochemical staining of normal and PBK corneas. Monoclonal antibodies to β_6 integrin (A, B, C) and β -catenin (clone 14; D, E, F) were used to examine frozen sections of normal (A and D), PBK (B and E), keratoconus (C), or radial keratotomy (F) corneas. β_6 integrin is not revealed in normal corneas (A), but the PBK corneas stained positively with the respective antibody (B). There is a marked increase in staining for β -catenin in PBK cornea (E) compared with a normal one (D). As controls, keratoconus corneas (surgical specimens, as are the PBK corneas) stain negatively for β_6 integrin (C). The radial keratotomy cornea (autopsy specimen, as are the normal corneas) stained positively for β -catenin mostly in the epithelial plug area (F). e, epithelium; s, stroma. Scale bar, 40 μ m.

the staining of the epithelial cell membranes was stronger than in normal corneas (not shown here).

DISCUSSION

Many ocular disorders have no appropriate animal model, and investigators rely on surgical specimens that often contain very limited quantities of RNA. Whereas RT-PCR technology allows one to evaluate the relative expression of a particular gene product, no convenient method for screening multiple gene products simultaneously has been available. In this report, we examined the possibility of using PCR-amplified cDNA generated by the Smart cDNA synthesis protocol to use as a probe on high-density nucleic acid arrays. This protocol requires only nanogram amounts of total RNA isolated from the tissue and relies on PCR to generate enough full-length cDNA to label as a probe. However, there were major concerns with this protocol involving the known propensity of PCR to give rise to artifacts such as nonrepresentative amplification of the target cDNA and its preference for shorter versus longer substrates. The available literature did not provide validation of this technique using conventional northern blot analysis.

Therefore, such a validation was performed in the present study. As shown in Figures 1 and 2, our amplification protocol maintained the presence of cDNAs that are known to be present in low level, moderate size in the mRNA pool (RGGT) as well as those of low level, relatively large size (tenascin-C). Importantly, the sizes of the respective transcripts were the same as revealed by Northern blot analysis. Although one

cannot demonstrate conclusively that the proportion of each individual mRNA is maintained throughout the amplification, the examples tested here provided very similar data to those found with Northern blot analysis in terms of mRNA sizes and levels. This indicates that the cDNA generated with Smart cDNA synthesis technique maintains a proportional representation of the total RNA and therefore could be used reliably to analyze nucleic acid arrays instead of reverse-transcribed poly(A)⁺ RNA.

High-density nucleic acid arrays and Smart cDNA synthesis represent some of the latest technological advances. These arrays have hundreds to thousands of transcriptionally regulated cDNAs categorized by their functions, which allows the identification of differences between two sources of RNA (in this case, normal and diseased corneas). The cDNA pools from normal or PBK corneas were used here to probe the nucleic acid arrays, focusing the analysis on adhesion molecules. As indicated in Figure 3, these techniques demonstrated marked differences in gene expression for β_6 integrin and β -catenin. These differences were then confirmed by immunohistochemistry at the level of protein expression.

It is important to realize that this approach can only reliably evaluate qualitative alterations in the expression of the cDNAs present on the array and cannot reliably assess mutations, deletions, or insertions in a particular gene product. Nonetheless, these techniques can be extremely useful in identifying differences in gene expression when comparing biological samples containing very small amounts of RNA. Indeed, the Smart cDNA protocol is reported to require a minimum of 50 ng of total RNA. In the present study, it allowed us to use successfully the equivalent RNA present in one half of a single cornea. At the same time, 20 corneas in each group had to be pooled to generate a similar signal in the Northern blot analysis of corneal tissue, especially for low copy number transcripts such as tenascin-C. Thus, Southern blot analysis of PCR-amplified cDNA (virtual Northern) seems to be a reasonable substitute for Northern blot analysis when the amount of RNA is limiting. This is especially true for surgical human corneal specimens that contain significantly less RNA than needed for traditional northern blot analysis.

Postsurgical corneal edema as represented by PBK has been associated with initial dysfunction and/or damage to the corneal endothelium after cataract removal with or without placement of an intraocular lens.⁷⁻⁹ Later in the course of the disease, epithelial changes develop, including formation of fluid-filled blisters (bullae) under the corneal epithelium and subepithelial fibrosis.^{7,11} The results presented here demonstrate that the expression of important epithelial adhesion molecules is altered in PBK corneas.

The appearance of β_6 integrin mRNA and protein in PBK is interesting because this subunit is part of the $\alpha_v\beta_6$ integrin that mediates cell adhesion to vitronectin, fibronectin, and tenascin-C.^{29,32-34} Tenascin-C, although absent from normal corneas, appears in the epithelial basement membrane, stroma, and Descemet's membrane of PBK corneas.^{11,30,31} The distribution pattern of β_6 integrin in PBK corneas correlated with the pattern of tenascin-C (manuscript in preparation). Perhaps, β_6 integrin is produced in response to the deposition of tenascin-C to allow better attachment of the epithelium to the altered basement membrane. Another possibility is that this integrin is upregulated to increase adhesion of PBK corneal epithelium to the extracellular matrix as the disease

progresses. Conversely, β_6 integrin that can bind transforming growth factor- β latency-associated peptide, thereby activating this growth factor,³⁵ can then cause fibrosis and tenascin-C deposition.

Both at the mRNA and protein levels, PBK corneas demonstrated a slight decrease in β_4 integrin with no change in α_6 integrin. Typically, $\alpha_6\beta_4$ integrin is found in hemidesmosomes of the basal epithelial cells and serves to link keratin filaments to the extracellular matrix.³⁶ Of interest is the observation that knockout mice deficient in β_4 integrin develop normally, although they display a blistered skin phenotype.³⁷⁻³⁹ It is tempting to speculate that a decrease in β_4 integrin may contribute to the formation of corneal epithelial bullae observed in PBK. It is not clear why α_6 integrin did not change in PBK. Because $\alpha_6\beta_4$ integrin in corneal epithelium may be predominant compared with another α_6 -containing integrin, $\alpha_6\beta_1$,¹⁹ α_6 and β_4 should have changed coordinately in PBK, which was not observed. It is reasonable to suggest that an increase of β_1 integrin expression in PBK (identified by gene array analysis; not shown here) might increase the proportion of $\alpha_6\beta_1$ compared with $\alpha_6\beta_4$, resulting in no change of α_6 integrin expression.

The epithelium of the normal corneas displayed weak staining for β -catenin, but in the PBK corneas there was a bright staining of all epithelial cell layers. This could be because of the masking in normal corneas of epitopes for the monoclonal antibodies used. However, this seems unlikely because two different antibodies produced similar patterns. Because the immunostaining and gene array results both show low β -catenin in normal corneas and its increase in PBK corneas, this protein most probably is produced in small amounts in normal corneal epithelium and becomes elevated in diseased epithelium.

This notion is indirectly supported by the observed differences in E-cadherin expression in normal and PBK corneas (not shown here). E-cadherin belongs to classic cadherins that mediate Ca^{2+} -dependent adhesion between cells. In epithelial cells, it complexes with β -catenin or with γ -catenin/plakoglobin, each of which can also bind α -catenin that mediates binding of cadherin-catenin complex to the cytoskeleton.^{22,40} Transfection of E-cadherin gene to cadherin-deficient cells can induce upregulation of β -catenin.⁴¹ In normal human corneas, E-cadherin is expressed only in small amounts if at all (see Ref. 42 and unpublished observations). It may thus be suggested that elevated expression of E-cadherin in PBK corneas will increase the expression of β -catenin, which agrees well with our experimental data (see the Results section). Elevated levels of both E-cadherin and β -catenin in PBK epithelium may represent a compensatory attempt by the cells to increase cell-cell adhesion in conditions associated with corneal swelling and formation of bullae.

β -catenin has multiple functions and plays a central role in development.^{43,44} Primarily, this molecule serves to bridge cadherins (involved in specific cell-cell adhesion) to the microfilament cytoskeleton.^{22,44} In addition, β -catenin is known to be involved in signal transduction pathways. It can interact with various receptors (e.g., epidermal growth factor receptor⁴⁵) at the cell membrane and can be heavily phosphorylated by protein tyrosine kinases.⁴⁶ In the cytosol, β -catenin is found complexed with the tumor suppressor, adenomatous polyposis coli protein,^{47,48} and glycogen synthase kinase 3β .^{49,50} In the nucleus, it has been identified as a complex with various

transcription factors of the lymphoid-enhancing factor family.^{51,52} Therefore, increased expression of β -catenin in PBK corneal epithelium could affect not only the adhesive but also the migratory, proliferative, and biosynthetic properties of the epithelium. The precise role of the elevated expression of β -catenin and E-cadherin in PBK corneas warrants further study.

While this article was being reviewed, two reports appeared that either used Smart cDNA synthesis technology to generate probes for gene arrays⁵³ or evaluated virtual Northern blotting by conventional northern blot analysis.⁵⁴ Using different methods, both reports confirmed our data on the validity of Smart cDNA synthesis and virtual Northern for the analysis of differential gene expression in small tissue samples.

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