Postnatal Corneal Transparency, Keratocyte Cell Cycle Exit and Expression of ALDH1A1

James V. Jester,¹ Young G. Lee,² Jiying Huang,² Jennifer Houston,² Brook Adams,² H. Dwight Cavanagh,² and W. Matthew Petroll²

PURPOSE. Recent studies have shown that rabbit corneal keratocytes abundantly express two water-soluble proteins, transketolase (TKT) and aldehyde dehydrogenase class I A1 (ALDH1A1), in vivo and that these proteins may contribute to corneal transparency at the cellular level. The purpose of this study was to determine the relationship between the expression of these proteins and the development of postnatal corneal transparency.

METHODS. Rabbits 1 day to 42 days of postnatal age were evaluated by in vivo confocal microscopy (CM) to measure corneal epithelial thickness, stromal thickness, and corneal haze. Selected corneas were then processed for immunocytochemistry and Western and Northern blot analyses, to determine stromal cell density, cell cycle entry, and expression of ALDH1A1 and TKT.

RESULTS. Quantitative measurement of corneal haze showed that the postnatal cornea was hazy after birth and became transparent during the first weeks after eyelid opening. Development of transparency was associated with decreased cytoplasmic light-scattering from postnatal corneal stromal cells, with the appearance of nuclear light-scattering after eyelid opening. Four days after birth, stromal cell density decreased rapidly, and the cells became quiescent, showing decreased staining by Ki67, a cell cycle marker. Whereas expression of TKT showed a gradual increase after birth, ALDH1A1 showed a marked increase after eyelid opening, and the combined expression significantly correlated with the reduction in light-scattering by postnatal stromal cells.

CONCLUSIONS. The data suggest that development of postnatal corneal transparency is associated with decreased keratocyte density and quiescence and the expression of TKT/ALDH1A1.

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Materials and Methods

Animals

Timed-pregnant female rabbits were purchased from Myrtle’s Rabbitry (Thompson Station, TN). Pregnant females were checked daily, and the date of delivery of the neonates was recorded. The neonates were then killed at 1, 4, 8, 10, 12, 16, 20, 30, and 42 days after delivery. All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In study 1, corneas were evaluated by in vivo confocal microscopy (CM), to measure light-scattering, epithelial thickness, and stromal thickness. After in vivo CM, the corneas were collected and processed for measurement of stromal cell density, cell cycle entry, and phenotype in the same eyes. All animals were killed at the same time of day ±1 hour, to control for diurnal variation in tissue hydration. In study 2, groups of eyes were evaluated at the same time intervals by in vivo CM, and batches of eyes were collected and processed to determine the level of expression of crystallin protein by Western and Northern blot analyses.

In all experiments the corneal diameter was measured with calipers before in vivo CM.

In Vivo CM

Epithelial thickness, stromal thickness, and light-scattering from postnatal rabbit corneas were measured by in vivo CM, according to a published technique. Briefly, examinations were performed with an in vivo confocal microscope (Tandem Scanning Corp., Reston, VA) with a 24× surface-contact objective (numerical aperture, 0.6: working distance, 1.5 mm). The system design and use has been described in detail. Before examination, the eyelids were opened, if not already so, and a drop of 2.5% hydroxypropyl methylcellulose (Gonio-sol; IOLAB Pharmaceuticals, Claremont, CA) was placed on the in vivo CM objective tip to serve as an immersion fluid. Subsequently, confocal microscopy through focusing (CMTF) analysis was performed as a continuous, z-axis scan through the entire cornea, starting in front of the epithelium and ending below the endothelium, as described in detail.

Four consecutive forward CMTF z-scans were performed in the central cornea at each examination. Depth intensity profiles from the CMTF scans were generated, and epithelial thickness, stromal thickness, and light-scattering were measured as previously described.

Measurement of Stromal Cell Density

After in vivo CM, the rabbits were humanely killed by lethal injection of pentobarbital sodium (100 mg/kg body weight). The eyes were removed and the corneas fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS). Tissue was then rinsed in PBS for 5 minutes, and a 2-mm block was cut from the central cornea. The specimens were then washed in PBS for 3 minutes, extracted in cold acetone for 3 minutes, and rinsed in PBS for 3 minutes. To allow the identification of cell nuclei, each specimen was stained en bloc with 5 µg/ml propidium iodide for 10 minutes and rinsed in PBS for 3 minutes. Selected blocks were also stained with FITC conjugated phalloidin (Invitrogen Corp., Carlsbad, CA), to identify actin organization. The tissue block was then mounted in 50% glycerol/PBS on a Mylar Petri dish (Backhofer GmbH, Reutlingen, Germany) and then observed with a laser scanning confocal microscope (Leica, Heidelberg, Germany). Optical sections through the en bloc-stained tissue were taken at 2-µm intervals in the z-axis. All nuclei were then counted in the optical volume. To calculate stromal cell density and take into account any tissue swelling or thinning due to ex vivo tissue processing, the thickness of the corneal stroma from each eye, as determined by in vivo confocal microscopy just before death, was used, as previously reported.

Transmission Electron Microscopy

Two eyes for each developmental day were fixed in 2% glutaraldehyde in phosphate buffer (pH 7.2). Tissue was then processed for transmission electron microscopy through the Pathology Microscopy Facility, University of Texas Southwestern Medical Center. Thin sections were stained with uranyl acetate and lead citrate and viewed on a transmission electron microscope (1200 EX; JEOL, Tokyo, Japan).

Quantitative Assessment of Ki67 Staining

Cell cycling of stromal cells was assessed by staining tissue sections with antibodies to the nuclear protein Ki67, which is expressed in actively cycling cells. Corneas were embedded in optimal cutting temperature compound (Tissue Tek; Sakura Finetek, Torrance, CA) and snap frozen in liquid nitrogen. The frozen blocks were stored in an ultracold freezer and then sectioned on a cryotome (Reichert Jung 2800 Frigocut; Leica). The sections were then stained with monoclonal anti-Ki67 (clone MIB-1; Chemicon/Millipore) and goat anti-mouse IgG conjugated to FITC (Invitrogen). To quantify the number of cycling cells, three eyes from each postnatal day were frozen and sectioned. For each eye, three good sections were examined (Diaplan Fluorescent Microscope; Leitz, Wetzlar, Germany). Each section was sampled along five 0.31-mm-wide contiguous regions, for a total of 1.55 mm of linear distance along the central cornea. For each region the number of labeled cells was counted in the epithelium, stroma, and endothelium. In addition, the area of stroma sampled was also recorded. The total number of cells for the five regions was then calculated, and the density of labeled stromal cells determined by dividing the total number of cells by the area evaluated. For the epithelium and endothelium, the total number of cells counted along the 1.55 mm of cornea surface was then divided by the distance, to provide the number of labeled cells per linear millimeter of cornea. An average of the three slides was then recorded for each eye, and the means and SD calculated for the three eyes were evaluated for each postnatal day.

Isolation and Characterization of Stromal Cell Crystallin Proteins

Postnatal rabbit stromal cellular proteins were isolated from days 1 to 42 after birth. To minimize other cell contamination, the surface epithelium was removed from the cornea by scraping with a scalpel blade, and the endothelium was scraped with a cotton-tipped applicator. The stromal tissue was then digested for 4 hours in sterile 2.0 mg/ml collagenase (Invitrogen-Gibco, Gaithersburg, MD) and 0.5 mg/ml hyaluronidase (Worthington Biochemical, Freehold, NJ) in minimal essential medium (MEM; Invitrogen-Gibco) at 37°C. Cells that were released from the stromal tissue were then centrifuged, washed twice in PBS, sonicated in 1 ml of buffer (25 mM Tris-HCl [pH 7.4] with 1 mM EDTA, 1 mM EGTA, 10 mM dithiothreitol, 5 µg/ml anti-pain, 5 µg/ml pepstatin A, 5 µm/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and separated into water-soluble and insoluble fractions before the addition of final buffer containing 1% SDS. Samples (50 µg/lane) were then run on 10% SDS-polyacrylamide gels and stained with Coomassie blue. For immunoblot analyses, proteins from SDS-PAGE were transferred to nitrocellulose membranes and reacted with rabbit anti-mouse TKT antibodies previously shown to recognize mouse corneal epithelial TKT and a rabbit anti-human ALDH1A1 (the generous gift of Ronald G. Lindahl, Department of Biochemistry, University of South Dakota, Vermillion, SD), followed by staining with peroxidase-conjugated goat anti-rabbit IgG (Invitrogen).

Northern Blot Analysis

Corneal stroma was isolated as just described and extracted (RNAzol method with TRI reagent; Molecular Research, Inc., Cincinnati, OH). RNA was electrophoresed (10 µg/lane) and blotted according to published techniques. Blots were then hybridized with cloned cDNA probes derived by RT-PCR of ALDH1A1 from rabbits.

Statistics

Statistical analysis was performed with the aid of commercial software (Sigma Stat for Windows Version 1.0, SPSS, Chicago, IL). One-way
ANOVA or the Kruskal-Wallis test was used to compare data obtained on the various days after birth. Correlation between the two values was performed with Pearson product moment correlation.

RESULTS

Development of Transparency in Postnatal Rabbits

Rabbits were born with translucent corneas and progressively developed corneal transparency during the first weeks of life from postnatal day 1 (PN1) to PN42 as revealed by biomicroscopy. As shown in Figure 1A, at PN4 the corneas appeared markedly hazy, with no iris details detectable. By PN12 (the approximate time of eyelid opening) the corneas showed a marked increase in size with decreased haze and the appearance of some iris details (Fig. 1B). After eyelid opening, corneal haze disappeared, and by PN30 the eyes became biomicroscopically transparent (Fig. 1C).

These biomicroscopic changes paralleled changes in light-scattering from the corneal stroma as detected in anterior-posterior x–z projections of the in vivo confocal microscopic three dimensional (3-D) data sets. At PN4, the corneas were thin and generated marked light-scattering throughout the stroma (Fig. 1D). At the time of eyelid opening (PN12), the corneal stroma showed increased thickness, but continued the marked light-scattering (Fig. 1E). By PN30, light-scattering decreased to levels similar to that of the normal adult rabbit cornea (Fig. 1F).

Changes in overall light-scattering detected in the x–z reconstructions were associated with distinct changes in the localization of light-scattering from individual x–y optical planes. At PN4, x–y plane images showed clusters of punctuate and diffuse light-scattering structures, suggesting the presence of densely packed corneal fibroblasts (Fig. 1G, arrows). The dense packing of corneal fibroblasts in the stroma was confirmed by ex vivo confocal microscopy after en bloc staining for nuclei and actin (Fig. 1J). This pattern of light-scattering appeared to change by PN12 (eyelid opening), when light-scattering appeared to localize to nuclei within the stroma and more weakly from cell bodies (Fig. 1H). This change in light-scattering was also associated with what appeared to be a decrease in cell density and actin filament assembly in the corneal fibroblasts (Fig. 1K). When corneas became more transparent at PN30, light-scattering appeared to be localized only to the stromal cell nuclei, with little or no detectible scattering from the cell body (Fig. 1I). The changes in light-scattering between PN12 and PN30 were also associated with a marked decrease in both cell density and actin filament assembly, which became predominately cortical (Fig. 1L).

Whereas marked qualitative changes in light-scattering, corneal fibroblast density, and actin filament assembly were detected from PN1 to PN42, little change in the collagen organization was seen by transmission electron microscopy (Fig. 2). At PN4, corneal fibroblasts appeared flattened but contained abundant rough endoplasmic reticulum (Fig. 2A). Stromal lamellae while thinned (double arrowhead) appeared to contain a uniform population of collagen fibrils of similar size and
spacing (Fig. 2B). The corneal stroma of transparent corneas, PN30, appeared similar to hazy and translucent corneas, with flattened stromal cells containing abundant rough endoplasmic reticulum (Fig. 2C). Stromal lamellae appeared thicker (Fig. 2C, double arrowhead), but collagen size and spacing did not appear remarkably different (Fig. 2D).

Changes in Epithelial and Stromal Thickness, Light-Scattering, and Stromal Cell Density

Quantitative changes in epithelial and stromal thickness, light-scattering, and stromal cell density, as measured by in vivo and ex vivo confocal microscopy, are presented in Table 1 and Figure 3. During early postnatal development, both the epithelial and stromal thicknesses were significantly thinner at all time points than in PN42 corneas (Table 1). In the corneal epithelium, the rate of epithelial thickening appeared to be biphasic, with a more rapid rate of increase occurring between PN1 and PN12, followed by a substantially slower rate of increase from PN16 to PN42 (Fig. 3A). In the stroma, thickness increased rapidly between PN1 and PN12, after which growth appeared to plateau at PN16 and even to decrease at PN20 (Fig. 3B). Increasing growth of the stroma was then detected from PN30 to PN42. This pattern of increasing thickness before eyelid opening followed by stromal thinning was similar to that previously reported in developing postnatal mouse corneas.11

Quantitative measurement of corneal haze showed marked light-scattering at birth that significantly increased between PN1 and PN4 (Table 1, Fig. 3C; \( P < 0.05 \)). After PN4, light-scattering progressively declined until PN12 (eyelid opening) and then remained constant to PN20. After PN20, light-scattering again progressively declined to PN42, when it reached adult levels. By comparison, the density of stromal cells was highest at birth, declining significantly by PN8 (Table 1, Fig. 3D; \( P < 0.05 \)). Density then remained stable until eyelid open-

![Figure 2](image2.png)

**Figure 2.** Transmission electron micrographs of postnatal corneas from PN4 (A, B) and PN30 (C, D). Note that stroma lamellae appeared thinner at PN4 than at PN30 (A, C, double arrow), but otherwise appeared similar. Magnification: (A, C) 500 nm; (B, D) 200 nm.

![Figure 3](image3.png)

**Figure 3.** Postnatal changes in epithelial thickness (A), stromal thickness (B), stromal light-scattering (C), and stromal cell density (D). Gray vertical bar: day of eyelid opening.

Table 1. Changes in Epithelial and Stromal Thickness, Haze, and Keratocyte Density

<table>
<thead>
<tr>
<th>Age (d)</th>
<th>Sample Size (Rabbits, n)</th>
<th>Epithelial Thickness (μm)</th>
<th>Stromal Thickness (μm)</th>
<th>Corneal Haze (( U_{AUC} \times 10^5 ))</th>
<th>Stromal Cell Density (Cells/mm(^3) ( \times 10^3 ))</th>
<th>Single-Cell Light-Scattering (( U_{AUC}/Cell ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>17.8* ± 2.0</td>
<td>159.7* ± 13.9</td>
<td>10.9† ± 2.1</td>
<td>382.2* ± 68.4</td>
<td>13.1* ± 4.7</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>21.4* ± 2.9</td>
<td>165.5* ± 7.6</td>
<td>16.8† ± 2.7</td>
<td>366.0* ± 28.4</td>
<td>19.8* ± 4.0</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>28.5* ± 3.2</td>
<td>200.4* ± 33.9</td>
<td>9.8† ± 3.2</td>
<td>244.8* ± 31.0</td>
<td>15.0* ± 7.2</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>30.5* ± 1.7</td>
<td>214.5* ± 34.3</td>
<td>8.8† ± 2.4</td>
<td>229.2* ± 40.0</td>
<td>13.1* ± 4.8</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>33.2* ± 2.8</td>
<td>223.0* ± 6.7</td>
<td>6.0† ± 1.3</td>
<td>224.6* ± 8.5</td>
<td>8.4 ± 1.8</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>40.2* ± 5.3</td>
<td>224.1* ± 11.3</td>
<td>5.7† ± 3.0</td>
<td>169.3 ± 19.4</td>
<td>10.8 ± 5.8</td>
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<tr>
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<td>5</td>
<td>43.0* ± 3.4</td>
<td>210.6* ± 10.9</td>
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<td>150.6 ± 27.5</td>
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<tr>
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<td>5</td>
<td>45.1 ± 1.9</td>
<td>250.5 ± 19.3</td>
<td>2.4 ± 0.5</td>
<td>98.3 ± 9.9</td>
<td>6.8 ± 1.4</td>
</tr>
<tr>
<td>42</td>
<td>5</td>
<td>48.9 ± 1.3</td>
<td>261.4 ± 8.5</td>
<td>1.4 ± 0.8</td>
<td>82.3 ± 14.6</td>
<td>5.0 ± 3.7</td>
</tr>
</tbody>
</table>

Only one eye of each rabbit was evaluated, by in vivo confocal and laser scanning confocal microscopy.

\* \( P < 0.05 \), one-way ANOVA on ranks versus the control group (Bonferroni \( t \)-test), PN42. Corneal haze: one-way ANOVA on ranks pair-wise multiple comparisons (Bonferroni \( t \)-test).

† \( P < 0.05 \) compared with PN42 and ‡ \( P < 0.05 \) compared with all others.
ing at PN12, followed by a more gradual but significant decline to PN42. Overall, changes in stromal haze showed a significant positive correlation with changes in keratocyte density ($r = 0.919$) and significant negative correlations with epithelial thickness ($r = -0.898$) and stromal thickness ($r = -0.911$) based on Pearson product moment correlation ($P < 0.001$). However, it should be noted that the marked increase in light-scattering that occurred at PN4 was not associated with any major change in epithelial thickness, stromal thickness, or keratocyte density. Further, light-scattering appeared to remain constant from PN12 to PN20, whereas keratocyte density continued to decline, and stromal thickness showed noticeable thinning at PN20.

To evaluate corneal light-scattering as a function of cell density, light-scattering measurements were divided by the total number of cells in the same optical volume ($120 \times 120 \mu m \times$ the stromal thickness) from the same eye to provide a measure of single-cell light-scattering (UAUC/cell, Table 1). Of note, even though the density of cells was high early in postnatal development, light-scattering based on the number of stromal cells was significantly ($P < 0.05$) higher from PN1 to PN10, before eyelid opening. Light-scattering as a function of the number of cells appeared to remain elevated from PN12 to PN20 and then markedly decreased at PN30 and PN42.

Changes in Ki67 Staining during Postnatal Development

To evaluate the potential for cell proliferation, corneal sections were stained with antibodies to the nuclear antigen, Ki67, which is expressed in actively cycling cells and has been used to assess in situ corneal cell proliferative potential.\textsuperscript{34,35} Results are presented in Table 2 and Figure 4 for epithelial, stromal, and endothelial cell Ki67 staining. The corneal epithelium generally showed a constant level of cell staining throughout postnatal development from PN1 to PN42 (Fig. 4A). A spike in cell cycle entry was observed at PN8, but no consistent trend was identified, nor was any significant difference in data collected on the different days. In contrast, stromal cell Ki67 staining (Fig. 4B) showed peak labeling at PN4 that was significantly higher than that observed at all other days ($P < 0.05$). Furthermore, Ki67 staining showed significantly elevated labeling of the stroma out to PN10, just before eyelid opening. Endothelial cells also showed Ki67 staining before eyelid opening (Fig. 4C); however, because of the low level of cell proliferation detected, the changes were not significantly different compared with PN42. Nevertheless, the data suggest that corneal endothelial cells continue to divide and proliferate up to eyelid opening and then exit the cell cycle, similar to stromal cells.

**TABLE 2. Ki67-Stained Corneal Cells**

<table>
<thead>
<tr>
<th>Age (d)</th>
<th>Sample Size (Rabbits, n)</th>
<th>Epithelium (Cells/mm)</th>
<th>Stroma (Cells/mm$^2$)</th>
<th>Endothelium (Cells/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
</tr>
<tr>
<td>1</td>
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</tr>
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<td>21.2</td>
<td>57.1*</td>
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</tr>
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<td>3</td>
<td>19.8</td>
<td>6.1</td>
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</tr>
<tr>
<td>30</td>
<td>3</td>
<td>16.6</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td>42</td>
<td>3</td>
<td>23</td>
<td>0.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* $P < 0.05$ compared with PN42.† $P < 0.05$ compared with other times.

**FIGURE 4.** Postnatal changes in Ki67 staining of the corneal epithelium (A), stroma (B), and corneal endothelium (C). Gray vertical bar: day of eyelid opening.
The relationship between cell cycling and stromal light-scattering was also investigated using correlation and regression analysis, which showed a positive significant ($P < 0.001$) correlation between these two factors ($r = 0.918$). Stromal staining, which showed a significant peak at PN4, appeared to correlate with a peak in light-scattering at the same postnatal day (compare Fig. 3C with Fig. 4B). Stepwise linear regression analysis using all measured variables also showed that changes in light-scattering were best predicted by Ki67 labeling and keratocyte density with $R = 0.952$ ($P < 0.001$).

### Postnatal Expression of Crystallin Proteins

For the evaluation of the postnatal expression of crystallin proteins, groups of postnatal rabbits were killed at various times from day 1 to day 42 after birth, cells from the stroma were then isolated and water soluble proteins collected. As shown in Figure 5A, SDS-polyacrylamide gels stained with Coomassie blue showed a gradual increase in the level of expression of the 70-kDa water-soluble protein from day 1 to day 42. This change was confirmed by Western blot analysis and densitometry of proteins (Fig. 5A, bottom; Fig. 6) with the use of an antibody specific for TKT,23 which showed gradually increasing expression of TKT starting at PN4 that reach adult levels by PN30. More interesting, however, expression of the 54-kDa protein was barely detectable in SDS-polyacrylamide gels of proteins extracted from day 1 to day 20 (Fig. 5A). By day 30, a prominent band migrating at 54 kDa was observed, suggesting that expression of the 54kDa ALDH1A1 was not upregulated until after eyelid opening. This notion was confirmed by Western blot analysis and densitometry with antibodies specific for ALDH1A1 (Figs. 5A, 6), which showed that expression of ALDH1A1 remained constant up to day 12, except for increased expression detected at PN1, with a marked decrease observed at PN4. This pattern of having higher ALDH1A1 expression on PN1 than on PN4 was observed in two separate groups of rabbits. On PN16, after eyelid opening, expression of ALDH1A1 was noticeably increased, with in-creasing expression detected on PN20, PN30, and PN42 (Figs. 5A, 6). Unlike TKT, however, levels of ALDH1A1 in the postnatal rabbits never achieved those observed in adult rabbit eyes obtained from an abattoir (Fig. 5A, adult). Changes in message specific for ALDH1A1, as identified by Northern blot analysis (Fig. 5B), also indicated that expression was not elevated until after eyelid opening (PN16). When the amount of TKT and ALDH1A1 was calculated based on the percentage of total water-soluble protein, a marked increase was detected beginning at PN16 (Fig 6, 7% of total water soluble protein). At PN42, TKT and ALDH1A1 comprised 19% of total keratocyte water-soluble protein.

**Figure 5.** Expression of the rabbit crystallin proteins TKT (70 kDa) and ALDH1A1 (54 kDa) detected with (A) SDS-PAGE and Western blot and (B) Northern blot.

**Figure 6.** Changes in expression of TKT, ALDH1A1, and TKT+ALDH1A1 as a percentage of water-soluble protein. Expression was determined by relative densitometry compared with PN1 for Western blots with anti-TKT and anti-ALDH1A1 antibodies and densitometry of Coomassie blue-stained SDS-PAGE of the water-soluble protein fraction for TKT + ALDH1A1 combined. Grey vertical bar: day of eyelid opening.

### DISCUSSION

Although it has been widely stated that postnatal corneal transparency develops after eyelid opening,3,12 there have been no studies measuring the progression of transparency in the rabbit and few that have evaluated possible underlying mechanisms. In this study we quantitatively evaluated postnatal corneal development by temporally measuring corneal light-scattering as an indicator of transparency using in vivo confocal microscopy, and we correspondingly assessed epithelial thickness, stromal thickness, stromal cell density, cell cycle entry, and expression of corneal crystallin proteins. Our findings indicate that postnatal corneal transparency develops in two phases: first, an early phase occurring before eyelid opening that plateau from PN12 to PN16 and then a later phase after eyelid opening that extends from PN16 to PN42. Important concurrent changes during postnatal corneal development that may affect transparency included the finding that stromal cell density undergoes a similar biphasic change, with a rapid decline from PN1 to PN12, followed by a more gradual decline after eyelid opening to PN42 that significantly ($P < 0.001$) correlates with loss of light-scattering ($r = 0.919$). However, it is interesting to note that light-scattering, calculated based on the number of cells within the stromal volume, showed high single-cell light-scattering from PN1 to PN20 and significantly lower cell light-scattering from PN30 to PN42. In parallel, the number of actively cycling, Ki67-positive cells in the stroma was high before eyelid opening and low or absent after eyelid opening, also showing a significant ($P < 0.001$) correlation with light-scattering ($r = 0.918$). Finally, expression of the corneal crystallin proteins as the percentage of total water-soluble protein was low before eyelid opening and rapidly increased after eyelid opening. Overall, these data suggest that postnatal development leads to critical changes in cells contained within the corneal stroma, including decreased cell density, cell cycle exit or quiescence, and upregulation of crystallin protein expression, which together significantly predict decreasing stromal light-scattering and development of corneal transparency ($R = 0.952$, $P < 0.001$). Although these changes imply that postnatal stromal cell development is critical to achieving adult corneal transparency, the mechanisms underlying the relationship with light-scattering and how these...
relate to known changes in extracellular matrix organization and corneal endothelial function require further examination.

**Postnatal Stromal Light-Scattering: A Cellular Versus Extracellular Basis**

In vivo confocal microscopic images showing densely packed, highly light-scattering stromal cells in translucent corneas and sparsely packed, low light-scattering cells in transparent corneas provides the most direct evidence that postnatal light-scattering is predominantly caused by stromal cells. In addition, the high correlation between stromal cell density and light-scattering with decreasing density associated with decreasing light-scattering further supports this conclusion. Almost duplicate findings have been reported in postnatal mouse corneas, which also show light-scattering localized to stromal cells, biphasic development of corneal transparency, and stromal cell density changes that parallel changes in light-scattering.

Although the present study qualitatively evaluated only the extracellular matrix by TEM, no marked change was noted in the overall fibril size or spacing other than an increase in the collagen lamellar thickness. This result is consistent with a previous ultrastructural report on rabbit corneal stroma morphogenesis and a more recent X-ray diffraction study in which postnatal collagen fibril size and spacing were evaluated in wild-type and lumican-deficient mice. In the more recent study, lumican-deficient mice that have persistent corneal haze showed significant variation in fibril spacing, diameter, and organization, whereas wild-type mice showed very little change from PN8 to PN14. The only changes noted were a slight reduction in spacing from 65.4 to 61.4 nm and a slight increase in fibril size from 31.0 to 32.3 nm. While the changes were not commented on by the authors, it is not clear that such small changes would account for the more than 50% reduction in light-scattering that occurs in both wild-type mouse corneas, as reported earlier, and the rabbit cornea, as reported in this study over the same time frame (PN8–PN14).

Although no clear ultrastructural changes were noted in the extracellular matrix, measurement of stromal thickness identified a rapid increase followed by a marked decrease in stromal thickness centered on eyelid opening. These postnatal changes again parallel changes in stromal thickness already reported in postnatal mice and further suggest important developmental events that occur in the stroma before and after eyelid opening. Although there is no clear explanation of the change in stromal thickness, we have proposed previously that the increased rate of stromal thickening may be related to increased synthesis of keratan sulfate proteoglycans, as noted in studies measuring lumican expression and radio-labeled sulfate uptake. Increased synthesis of lumican and keratan may then lead to swelling of the cornea and increased thickness before eyelid opening, whereas decreased thickness after eyelid opening may be explained by evaporation and endothelial pump function. Such a hypothesis is also consistent with the observation that rapid stromal thickening before eyelid opening is not detected in lumican-deficient mice that have markedly reduced synthesis of both lumican and keratan because of the dependence of keratan expression on lumican gene expression. However, as mentioned, X-ray diffraction studies have failed to detect any major change in fibril spacing during this phase of postnatal development. Furthermore, the decrease in fibril spacing noted by X-ray diffraction (~1.6 nm) could not account for the stromal thinning of 12 μm that was detected in the mouse from PN12 to PN14.

Alternative explanations that have been proposed are the formation of collagen-free lakes similar to those proposed by Benedek to explain corneal haze after swelling, and increased cellular volume. Neither of these explanations appears satisfactory, since the formation of collagen-free lakes should lead to increased stromal light-scattering, and increased stromal cell volume should lead to increased cell density, neither of which was detected during this phase of postnatal development. Although the reason for postnatal stromal thickening and thinning remains unclear and requires further study, the lack of any clear association with light-scattering (i.e., an increase in light-scattering during stromal thickening from PN8 to PN12, or an accelerated transparency during stromal thinning from PN16 to PN20) suggests that the changes in stromal thickness and extracellular matrix are unrelated to the development of transparency at this postnatal stage.

**Keratocytes, Crystallins Proteins, and Corneal Transparency**

In the present study, early postnatal corneas that showed a high level of light-scattering contained a high density of actively cycling stromal cells that had abundant rough endoplasmic reticulum, prominent actin filaments, and expressed low levels of the corneal crystallin proteins TKT and ALDH1A1. The development of postnatal transparency, however, was associated with decreased stromal cell density, cell cycle exit, decreased actin filaments, and increased expression of corneal crystallin proteins after eyelid opening. Although the density of stromal cells showed a high correlation with stromal light-scattering, the changes in cell density alone did not appear to explain the development of postnatal transparency. When light-scattering was calculated as a function of the number of stromal cells in the same optical volume, early in postnatal development stromal cells had significantly higher levels of light-scattering than cells at later postnatal ages, particularly after eyelid opening. Although subtle changes in the extracellular matrix organization may explain these differences, alternatively this finding suggests that factors other than cell density play a role in the development of postnatal transparency—particularly the differentiation of the stromal cells to an adult keratocyte phenotype.

It is interesting to note that early postnatal stromal cells show morphologic and biochemical features similar to those of corneal wound-healing fibroblasts, which also markedly scatter light. This similarity has been commented on by Cintron et al., who noted that both show a low cell-to-collagen ratio and abundant rough endoplasmic reticulum. Corneal fibroblasts also retain the developmental program for synthesizing and depositing uniform-sized, orthogonally arranged collagen matrix when cultured or ectopically transplanted, but are uniformly associated with regions of haze and corneal opacification. Cultured corneal fibroblasts and wound-healing fibroblasts also show high rates of proliferation, decreased expression of keratan sulfate proteoglycans, and decreased expression of corneal crystallin proteins. Phenotypic modulation of rabbit keratocytes in culture that induce cell proliferation, actin filament assembly, and decreased expression of TKT and ALDH1A1 also increase single-cell light-scattering, suggesting a connection between these variables. Together, these findings indicate that there are distinct morphologic and biochemical similarities between early postnatal stromal cells, wound healing fibroblasts, and cultured corneal fibroblasts, all of which are detected in opaque or translucent tissue, which distinguishes them from adult corneal keratocytes that are present in transparent corneas. The differences between corneal fibroblasts and keratocytes therefore may underlie the differences in light-scattering detected in vivo and in vitro (i.e., cell cycle entry, actin filament organization, and crystallin protein expression).

Although the presence of abundant actin filaments in corneal fibroblasts may seem a likely cause of cellular light-scatter-
tering, it is not clear why keratocytes abundantly express a few water-soluble proteins to such high levels, exceeding 60% of total water-soluble protein in some species. More recent studies also indicate that overexpression of ALDH3A1 in human corneal epithelial cells and keratocytes is protective against various forms of UV and oxidative stress. ALDH1A1 has been shown to have metabolic functions similar to those of ALDH3A1 and is capable of metabolizing hexanal, 4-hydroxynonenal, and malondialdehyde, the major products of lipid peroxidation. It is likely therefore that protection against these forms of stress are critical to the maintenance of corneal transparency, by reducing oxidative free radicals that lead to lipid peroxidation and the formation of lipid–protein aggregates that may markedly scatter light.

More interesting, recent studies by Pappa et al. have shown for the first time a close link between the expression of the crystallin protein ALDH3A1 and cell proliferation, with overexpression leading to the slowing of cell cycle progression in cultured human corneal epithelial cells. Since ALDH3A1 is also expressed in human keratocytes, a similar effect on cell cycle progression may be expected for all corneal cells abundantly expressing this protein. In addition, inhibition of cell proliferation has been shown to result in maintenance of abundant crystallin protein expression. A similar relationship was identified in the present study, with ALDH1A1 expression appearing to correlate inversely with cell cycle progression. Of particular note, at PN4, levels of ALDH1A1 expression showed a marked decrease at the same time that keratocytes showed a significantly more Ki67 staining, cell cycle entry, and cellular light-scattering. A similar relationship was detected at later stages of postnatal development when levels of ALDH1A1 expression increased after stromal cells apparently exited the cell cycle. Although additional studies are needed to determine whether ALDH1A1, like ALDH3A1, directly affects cell cycling, the data strongly suggest that there is an inverse relationship between the expression of crystallin proteins and cell proliferation. Based on these data, we hypothesize that the abundant expression of ALDH1A1 in the rabbit is a marker for the adult keratocyte phenotype and plays an important role in the maintenance and/or development of cellular transparency.

References


