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Corneal Keratocytes: Phenotypic and Species Differences in Abundant Protein Expression and In Vitro Light-Scattering

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Abstract

PURPOSE—Previous studies suggest that corneal haze after injury involves changes in the light-scattering properties of keratocytes that are possibly linked to the abundant expression of water-soluble proteins. The purpose of this study was to determine the protein expression pattern of keratocytes from different species and different cultured rabbit keratocyte phenotypes and to assess differences in light-scattering in vitro.

METHODS—Water-soluble proteins were isolated from corneal epithelial cells and keratocytes of several species, including human (Hu), mouse (Mo), rabbit (Ra), chicken (Ch), and pig (P) and different cultured rabbit keratocyte phenotypes. Proteins were then characterized by SDS-PAGE, tryptic peptide sequence analysis, and Western blot analysis. Light-scattering and actin organization from cultured cells were determined with confocal reflectance and fluorescence microscopy, respectively.

RESULTS—Protein expression patterns varied substantially between species and cell types, with five new abundantly expressed proteins identified including, LDH (Ra, Ch), G3PDH (Hu, Ch), pyruvate kinase (Ch), Annexin II (Ch), and protein disulfide isomerase (Ch). Different rabbit keratocyte phenotypes also showed different levels of expression of ALDH1A1 and TKT, with myofibroblasts showing the greatest reduction. Myofibroblasts showed significantly greater ($P < 0.05$) light-scattering but also showed the greatest organization of actin filaments.

CONCLUSIONS—Abundant protein expression is a characteristic feature of corneal keratocytes that is lost when cells are phenotypically modulated in culture. Greater light-scattering by myofibroblasts also provides support for a link between cellular transparency and haze after injury that is possibly related to loss of protein expression or development of prominent actin filament bundles. (*Invest Ophthalmol Vis Sci.* 2005;46: 2369-2378) DOI:10.1167/iovs.04-1225

Past studies have demonstrated that the cornea abundantly expresses a few cytosolic enzymes and proteins, which as a class have been popularly referred to as corneal crystallins, based on the similarity in the level of expression to lens enzyme crystallins.¹⁻³ Although the exact function of corneal crystallins remains undetermined, it has been hypothesized that they play a role in the development and/or maintenance of tissue transparency through their effects on

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light-scattering and absorption,^{4,5} as well as their metabolic roles in mitigating the effects of oxidative and UV light stress.^{6,7}

The first recognized abundantly expressed water-soluble corneal protein identified was BCP 54 (bovine corneal protein, molecular mass, 54 kDa),⁸ which has been identified as aldehyde dehydrogenase class 3 (ALDH3A1),^{6,9,10} and comprises up to 20% to 40% of the total water-soluble protein extracted from the intact bovine cornea. Other corneal crystallins that have been identified include, transketolase (TKT) in mouse, rabbit, and human corneas^{11,12}; aldehyde dehydrogenase 1A1 (ALDH1A1) in the rabbit cornea¹²; α -enolase in human, mouse and chicken corneas¹³; isocitrate dehydrogenase in the bovine cornea¹⁴; peptidyl-prolyl *cis-trans* isomerase and argininosuccinate lyase in the chicken cornea¹³; glutathione *S*-transferase-related protein in the squid cornea¹³; and gelsolin and actin in the fish cornea.¹⁵ The prevalence of TKT and ALDH1A1/3A1 expression in mammalian corneas contrasted with their absence in other species, such as chicken and fish, suggests taxon-specific expression patterns for these abundantly expressed proteins similar to lens enzyme crystallins.

For the most part, the identification of corneal crystallins has focused on the analysis of water-soluble protein extracts from whole corneas or isolated corneal epithelium. More recently, abundantly expressed proteins have been identified in isolated rabbit corneal keratocytes and proposed to play a role in regulating corneal transparency at a cellular level.¹² Protein extracts of keratocytes isolated from transparent rabbit corneas showed abundant expression of TKT and ALDH1A1, whereas extracts of repair fibroblasts from injured and hazy corneas showed marked reductions in the expression of these proteins. In addition, serum-cultured keratocytes, which mimic corneal wound-healing fibroblasts, also show marked reductions in both ALDH1A1 and TKT,^{17,18} which has been associated with ubiquitin-proteasome degradation.¹⁹ These findings, taken together with objective light-scattering measurements obtained by in vivo confocal reflectance microscopy showing prominent backscattering of light from wound-healing fibroblasts and myofibroblast,^{12,20} suggest that these proteins play an important role in controlling light-scattering from cells within the corneal stroma.

To extend our knowledge of keratocyte crystallins, we analyzed the expression of water-soluble proteins extracted from isolated corneal keratocytes and compared their expression pattern to that of the corneal epithelium for a range of different species including rabbit, human, mouse, pig, and chicken. We have also established phenotypically modulated rabbit corneal keratocytes, determined the water-soluble protein expression pattern, and measured the single-cell light-scattering for each phenotype. Keratocytes characteristically show abundant expression (>3.5% of total) of a few water-soluble enzymes and proteins, although there are distinct differences between species. These studies also establish for the first time that cultured myofibroblasts show significantly greater single-cell light-scattering than do either fibroblasts or keratocytes, while expressing the lowest levels of TKT and ALDH1A1.

METHODS

Tissue Collection

Rabbit, pig, and chicken eyes were obtained from Pel Freez (Rogers, AR). Eyes were shipped overnight in minimum essential medium (MEM), received, and processed within 24 hours after enucleation. Human corneas were obtained fresh from the Eye Bank at the University of Texas Southwestern Medical Center. Mouse eyes were obtained fresh from mice after cervical neck dislocation of sedated animals (halothane). Handling and treatment of mice and human tissue conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and adhered to the tenets of the Declaration of Helsinki.

Collection of Keratocytes, Epithelium, Endothelium, and Scleral Fibroblasts

Epithelial cells were scraped from the corneal surface of intact eyeballs with a no. 10 Bard Parker blade, and samples were immediately placed in cold (4°C) extraction buffer (E-buffer) containing 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 µg/mL antipain, 5 µg/mL pepstatin A, and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO). Corneas were then removed from the eyeballs and placed in sterile MEM.

To collect corneal endothelium, Descemet's membrane was peeled from the corneal buttons and placed in collagen digestion buffer (CD-buffer) containing 2.0 mg/mL collagenase and 0.5 mg/mL hyaluronidase in MEM (Invitrogen-Gibco, Grand Island, NY). The membranes were then incubated at 37°C until fully digested (~4 hours). The corneal endothelial cells were then centrifuged, the pellet washed three times in MEM, and the cells suspended in E-buffer at 4°C.

After the corneal epithelium and endothelium had been removed, corneal keratocytes were isolated by digesting corneal buttons in CD-buffer for 4 hours at 37°C. Keratocytes were then spun down and the cells washed three times in MEM. The final pellet was resuspended in E-buffer at 4°C.

Cell samples from scleral tissue were also obtained by first removing the overlying conjunctiva. Any attached retina and underlying choroid was then removed by scraping the internal side of the sclera with a scalpel blade. The sclera was then digested for 4 hours in CD-buffer at 37°C, and the cells were collected, washed, and resuspended in E-buffer at 4°C.

Culture of Rabbit Keratocyte Phenotypes

Keratocytes in the rabbit eyes were isolated according to previously described techniques.²¹ Cells were cultured in DMEM containing 1% RPMI vitamin mix, 100 µM nonessential amino acids, 1 mM pyruvate, 100 µg/mL ascorbic acid, and 1% penicillin-streptomycin-amphotericin B (all from Invitrogen Corp., Carlsbad, CA) at 37°C in a humid atmosphere containing 5% CO₂ at a density of 1.0×10^4 cells/cm² for all experiments. Modulation of the keratocyte phenotype was accomplished by culturing keratocytes in specific growth factor-supplemented media, as described previously, to generate fibroblast and myofibroblast phenotypes.²² Specifically, keratocytes were grown in serum-free culture medium alone or in media supplemented with 1.0 ng/mL insulin-like growth factor (IGF)-II (Invitrogen-Gibco), 100 ng/mL platelet-derived growth factor (PDGF)-AB (Upstate Biotechnologies, Lake Placid, NY), 10 ng/mL FGF2 (Invitrogen Corp.), and 1 ng/mL TGFβ1 (Sigma-Aldrich). Cells were cultured for 7 days in 10-cm dishes (Corning Costar, Corning, NY) to allow for the development of specific phenotypic characteristics and then passed onto either 25-mm coverslips or 10-cm dishes. Cells were allowed to attach overnight and then assessed for in vitro light-scattering or collected in E-buffer for biochemical analysis.

Extraction of Water-Soluble Proteins

Isolated corneal epithelial cells, endothelial cells, keratocytes, scleral cells, and cultured rabbit keratocyte phenotypes in E-buffer were sonicated at 4°C for less than 1 minute and then stored for 1 hour on ice to extract water-soluble proteins. Samples were then centrifuged at 12g for 3 minutes and the supernatant collected. For collection of crystallin proteins from the lens and retina of rabbit, tissues were sonicated in E-buffer directly, without isolation of cells. Samples were then centrifuged and the water-soluble fraction collected. The amount of protein was determined by a protein assay modified for use with thiols (DC assay; Bio-Rad Laboratories, Hercules, CA).

Identification and Characterization of Water-Soluble Proteins

Water-soluble protein extracts (30 µg/lane) were run on 10% polyacrylamide gels and stained with Coomassie blue. Protein gels were then digitally scanned (Power LookII; Umax Data Systems, Inc., Industrial Park, Taiwan), and the density of each lane and each major band were measured with gel scanning software (1-D Gel Scan; MetaMorph; Universal Imaging Corp., Downingtown, PA). The percentage contribution of each major band to the total water-soluble protein for that sample was then determined by dividing the density of each band by the total density of each lane, after adjusting for background. Because individual proteins are compared to the total water-soluble protein level in each sample, providing an internal standard, minor differences in loading densities between samples are automatically corrected. In addition, when comparing cultured cells, calculating the percentage of water-soluble protein takes into account differences that may arise in cell volume between different keratocyte phenotypes that may influence the density of individual bands when comparing across cell phenotypes.

Major water-soluble proteins that comprised greater than 3.5% of the total water-soluble proteins were submitted for peptide sequence analysis to the Howard Hughes Medical Institute Biopolymer Facility at the University of Texas Southwestern Medical Center. Coomassie bluestained polyacrylamide gels were provided to the facility, and the major bands were removed and processed on an ion-trap mass spectrometer (LCQ-DECA; Finnigan, San Jose, CA) equipped with an online capillary HPLC system (Waters, Milford, MA). Sequences were checked for homology to known protein sequences. The identity of selected proteins was then confirmed by Western blot analysis using rabbit anti-human ALDH1A1 (a gift from Ronald Lindahl, Department of Biochemistry, University of South Dakota, Vermillion, SD), rabbit antimouse TKT (a gift from Joram Piatigorsky, National Eye Institute, Bethesda, MD), rabbit anti-human ALDH3A1 (Ronald Lindahl), goat anti-human annexin II (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-glyceraldehyde 3 phosphate dehydrogenase (G3PDH; Trevigen, Gaithersburg, MD).

Measurement of Light-Scattering from Single Cells

Cells were plated onto 25-mm diameter glass coverslips with a 10 mm diameter, 50-µm-thick acrylamide gel-collagen substrate attached (Fig. 1). Attachment and coating of the acrylamide gels to the glass coverslips were performed according to the methods previously describe by Pelham and Wang.²³ Before the cells were plated, the coverslips were washed and soaked in DMEM for 30 to 45 minutes. Cultured cells were then plated onto the collagen-coated gels at a density of 2.0×10^5 cells/cm², in appropriate culture medium and allowed to attach overnight. This process produced a single cell layer of attached cells of moderate density (50%).

Coverslips were then placed in the base ring of the artificial stromal chamber (Fig. 1), the gasket fitted, and the chamber filled with appropriate medium supplemented with 50 mM HEPES buffer to help maintain pH. The chamber was then closed with the locking ring and placed on a heat block to maintain temperature at 37°C. The artificial stromal chamber and heat block were then positioned in front of an in vivo confocal reflectance microscope (Tandem Scanning Corp., Reston, VA), such that the tip of the objective was in contact and completely perpendicular to the surface of the glass coverslip containing the collagen-coated gel and attached cells. Confocal microscopy through-focusing (CMTF) data sets were then collected, as previously described,²⁴⁻²⁶ except that the lens advance speed was reduced to 20 to 40 µm/s, providing a 0.33- to 0.66-µm separation between individual images in the three-dimensional (3-D) data set. At least three CMTF data sets were taken from three different regions of each coverslip, and there were two to three coverslips for each condition evaluated. In a single experiment, all five keratocyte phenotypes were tested on the same day, all originating from the same initial culture. Only one coverslip was mounted in the artificial stromal chamber at a time and then evaluated immediately after mounting. After scanning, coverslips were removed

from the artificial stromal chamber, and the cells were fixed in paraformaldehyde and stained with FITC-conjugated phalloidin (Molecular Probes, Eugene, OR), as previously described.²²

To measure light-scattering from single cells, each 3-D data set containing 200 sequential images was imported into image-processing software (MetaMorph; Universal Imaging Corp.). Individual cells were then outlined and the cell area determined. For each plane within the 3-D data set, the total pixel intensity within the region of interest or cell area was determined for the three cells contained within each data set. For each cell, the average pixel intensity per square micrometer was then calculated by dividing the total pixel intensity measured by the cell area for each plane, and the results were plotted as a function of axial depth. The area under the curve was then integrated as a measure of total light-scattering from each cell (U_{auc}). Three cells from each 3-D data set were measured and the average $\pm$ SD of 18 to 27 cells from two to three different coverslips was recorded in each condition. In general the methods used to quantify light-scattering by individual cells used established methods for measuring light-scattering from corneas.²⁶

Validation of Light-Scattering Measurements using Confocal Reflectance Microscopy

To validate the light-scattering measurements obtained from the in vivo confocal reflectance microscope, phantom corneas of known light-scattering properties were fabricated with polyacrylamide gels containing different concentrations of formazin, a standard light-scattering solution that is used in turbidimetry measurements. Formazin is a submicron polymer suspension of microspheres in ultrapure water with a particle size distribution between 0.02 and 0.203 μm . A standard 1,000 NTU (nephelometric turbidity unit) solution of formazin was centrifuged at 3,000 rpm for 30 minutes and the pellet resuspended in 30% acrylamide to yield a final concentration of 12,000 NTU in 12% acrylamide. This solution was then further diluted to 9000, 6000, 3000, and 0 NTU with 12% acrylamide. Phantom corneas were then constructed by forming acrylamide gels of 150- μm thickness from the formazin-acrylamide solutions. Transparent gels were also formed of 150- and 800- μm thickness prepared from 12% acrylamide solution without formazin. Formazin-acrylamide gels were then sandwiched between the transparent gels and mounted in the artificial stromal chamberlike device. Using the transparent gels as spacers for the formazin-acrylamide gels separated the light-scattering signal observed from the coverslip glass-liquid interface from the formazin-acrylamide signal. These phantom corneas were then scanned by confocal reflectance microscopy, and the light-scattering measured according to published techniques.²⁴⁻²⁶

Statistical Analyses

All statistical comparison were made using SigmaStat 1.0 (SPSS Science, Chicago, IL). Comparison between groups for analysis of light-scattering by different keratocyte phenotypes and different phantom corneas were performed with a Tukey all-pair-wise, multiple-comparison procedure and the Dunnett method of multiple comparisons versus a control group one-way analysis of variance.

RESULTS

Rabbit Ocular Crystallin Proteins

Water-soluble protein extracts from the corneal epithelium, keratocytes, and endothelium showed distinct differences and similarities based on cell type (Fig. 2A). In the corneal epithelium, 5 proteins (I-V) were identified that had expression levels ranging from 4.3% to 14.7% of the total water-soluble protein (Table 1). The most abundant protein was lactate dehydrogenase B (LDH, band V) representing a total of 14.7%, followed by TKT at 6.9% (band I), glyceraldehyde 3 phosphate dehydrogenase (G3PDH) at 6.9% (band IV), α -enolase at 4.5%

(band III), and ALDH1A1 at 4.3% (band II). In contrast, the most abundant water-soluble protein expressed by keratocytes was TKT (14%) and ALDH1A1 (12.7%), with much lower levels of LDH (3.8%) and G3PDH (3.8%) compared with the epithelium. α -Enolase was barely detectable in the keratocyte water-soluble fraction. Corneal endothelial cells showed a water-soluble protein expression profile that was essentially identical with that of the corneal keratocyte, with TKT and ALDH1A1 representing the most abundantly expressed water-soluble proteins.

Crystallin proteins identified from other ocular tissues obtained from the rabbit eye were compared to keratocyte crystallins (Fig. 2B, lane 1). As previously identified, prominent expression of a few water-soluble proteins was not detected in the sclera (lane 2).¹² Of particular interest was the finding that scleral cells expressed levels of G3PDH almost equal to those of corneal keratocytes and lens protein extracts, yet the levels of TKT and ALDH1A1 from keratocytes were markedly higher than in scleral cells, based on Western blot analysis. Although the general pattern of protein expression may appear similar, the use of G3PDH as a standard for comparison shows the levels of TKT and ALDH1A1 to be obviously different.

Extracts from the whole retina (Fig. 2B, lane 3) showed high expression for G3PDH (band IV) and α -enolase (band III). Unlike the cornea, sequence analysis showed the 54-kDa band migrating with corneal ALDH1A1 to be ALDH3A1 in the retina, which was later confirmed by Western blot analysis (data not show). In addition to the expression of lens-specific crystallin proteins (Fig. 2B, lane 4), extracts from the rabbit lens showed high levels of expression for both G3PDH (band IV) and LDH (band V).

Corneal Crystallins from Other Species

Water-soluble protein extracts from human, mouse, chicken, and pig corneas were also evaluated (Fig. 2C) and the abundant proteins in both corneal epithelium (lane 1) and keratocytes (lane 2) compared by SDS-PAGE and Western blot analysis for TKT, ALDH1A1, and ALDH3A1. Similar to the rabbit, five major water-soluble proteins were identified in the human cornea: TKT, ALDH1A1/3A1, α -enolase, G3PDH, and LDH. Although the profiles from the corneal epithelium and keratocytes were generally similar, slight differences in the percentage contribution of the various proteins were noted, for instance TKT (13.7%) was the most prominent water-soluble protein in the corneal epithelium, whereas ALDH1A1/3A1 (14.7%) was more prominent in the keratocytes (Table 1). Different from the rabbit, human cornea expressed approximately equal amounts of both ALDH1A1 and ALDH3A1. In the mouse, keratocytes expressed predominantly TKT and ALDH3A1, with small amounts of ALDH1A1. This pattern was similar to that in the mouse corneal epithelium, although the epithelium also expressed high levels of actin (band VI, 4.0%) and glutathione *S*-transferase (band VII, 4.2%), which were not abundantly expressed (>3.5%) in the keratocytes. In the pig cornea, the most abundantly expressed water-soluble protein in the epithelium was ALDH3A1, which comprised almost 33.0% of the total water-soluble proteins. Unlike the epithelium, keratocytes expressed even greater amounts of ALDH, comprising 55.0% of the total water-soluble proteins, with equal contributions of ALDH1A1 and 3A1. The keratocytes also abundantly expressed LDH (8.0%), resulting in more than 63% of the total water-soluble protein comprising only two cytosolic enzymes. Although the pig corneal epithelium also expressed LDH and other minor proteins, the amount of these proteins was not greater than the 3.5% corneal crystallin protein cutoff.

Unlike the water-soluble proteins from mammalian corneas, chicken corneas did not express high levels of ALDH, although ALDH1A1 was detectable by Western blot analysis (Fig. 2C). Overall, the chicken cornea appeared to express the most diverse set of corneal crystallin proteins including TKT, α -enolase, G3PDH, and LDH, all of which were expressed at abundant levels in the mammalian corneas, as well as pyruvate kinase, protein disulfide

isomerase, and annexin II. Comparing the keratocytes to the corneal epithelium also showed differences in the levels of abundance of these proteins, with TKT (11.5%) representing the most abundant protein in the chicken keratocytes contrasted with G3PDH (7.5%) and protein disulfide isomerase (7.4%) in the corneal epithelium.

Validation of Light-Scattering Measurements

To assess the ability of in vivo confocal reflectance microscopy to detect differences in light-scattering, phantom corneas were prepared from acrylamide gels that contained different concentrations of formazin. The use of formazin to standardize in vivo confocal reflectance microscopy measurements of light-scattering has been reported for solutions (Shaver JH, et al. *IOVS* 2002;43:ARVO E-Abstract 1709). Assessment of light-scattering from the different solutions was difficult because of strong light-scattering at the tip of the microscope objective lens, where differences in the refractive index from glass to water produced a strong backscattering signal. To remove this background noise, corneal phantoms were fabricated, comprising three layers of transparent acrylamide, the middle having a 150- μ m-thick acrylamide gel composed of different concentrations of formazin to simulate regions of wound-healing fibroblasts.

It should be noted that a standard solution of 1000 NTU formazin that is 1 cm thick, as used in standard turbidimetry, is completely opaque and does not transmit light. However, fabricated gels that are only 150 μ m thick appear transparent and have a predicted light transmission of 94%. An entire cornea (~500 μ m thick) that is comprised of light-scattering particles with a density similar to 1000 NTU formazin, would still have a light transmission of 80%. To generate a broader range of different light-scattering gels with marked variation in light transmission, 1,000 NTU formazin was concentrated and reconstituted in acrylamide at a concentration equivalent to 12,000 NTU. Predicted light transmission through a 150- μ m acrylamide gel containing 12,000 NTU formazin was calculated to be 46%. Light transmission through the other gels was calculated to be 56% for 9000 NTU, 68% for 6000 NTU, and 82% for 3000 NTU.

Formazin within the acrylamide gel showed some clumping of particles, particularly at the higher concentrations, as seen by confocal reflectance microscopy (Fig. 3A). Clumping produced pockets of transparent gel interspersed with regions of light-scattering, simulating the light-scattering patterns detected in corneal wounds. In reconstructions of the CMTF data sets, strong backscattering signals were obtained from the surfaces of the glass coverslip (Fig. 3B, arrow). The anterior transparent gel effectively moved the light-scattering gel away from the strong signal of the glass coverslip. In most preparations, it was not possible to detect the surface of the anterior gel, either due to the strong signal from the coverslip, or due to the completely transparent nature of the gel. Occasionally, fine particles on the surface of the gel made it possible to detect the gel surface. The interface between the formazin gel and the anterior and posterior transparent gels were easily detectable looking at the XZ projections from the CMTF scans (Fig. 3B), which also showed clumping of formazin within the gel and particularly along the anterior and posterior surface of the gel. Pixel intensity curves (Fig. 3C) also showed easily detectable peaks originating from the glass coverslip (arrow) and the formazin gel, which were quantified by measuring the area under the curve to obtain a measure of overall light-scattering from the formazin-containing gel. It should be noted that there was some attenuation of signal, as detected by a downward slope of the curve as the focal plane passed through the gel. This was particularly noticeable for the highest concentration of formazin (i.e., 9,000 -12,000 NTU) and was similar to that which has been observed in very edematous and opaque corneas.

A representative experiment measuring light-scattering from the formazin gels (3,000 -12,000 NTU) compared with a transparent middle gel of the same thickness (0 NTU) is shown in

Figure 3D. There was a significant difference ($P < 0.001$) associated with the effect on measured light-scattering and the concentration of formazin. In general, the relationship appeared linear, with a correlation of $r^2 = 0.92$. This experiment was repeated at least three times, and all experiments showed similar relationships, indicating that differences in the measured area under the curve obtained by the *in vivo* confocal reflectance microscopy CMTF approach were related to differences in the light-scattering of the sample.

Expression of Crystallin Proteins by Different Rabbit Keratocyte Phenotypes

The phenotype of cultured rabbit keratocytes was modulated by growth in serum-free media supplemented with growth factors known to alter the contractile and protein expression pattern of keratocytes (grown under serum-free or IGF-II conditions) to fibroblasts (FGF2 and platelet-derived growth factor [PDGF]) or myofibroblasts (TGF β).^{22,27,28} As discussed in the Methods section, essentially the same cells from the same batch of identically treated corneas were evaluated for both light-scattering measurements and expression of water-soluble proteins. The level of water-soluble proteins expressed by different keratocyte phenotypes identified by SDS-PAGE and Western blot analysis are shown in Figure 4A and 4B for equal loads (30 μ m per lane). Note that keratocytes isolated from intact corneas with only 4 hours of digestion (lane 1) express high levels of both TKT and ALDH1A1 representing 19.1% and 14.5% respectively of the total water-soluble proteins collected. Keratocytes cultured for 7 days and passaged overnight in serum-free medium (lane 2) showed a marked loss in the expression of ALDH1A1 that measured 3.8% of the total water-soluble protein or 26% of the level detected *in situ*. Levels of TKT in serum-free cultured keratocytes also decreased to 49% of that expressed by *in situ* keratocytes or 9% of the total water-soluble protein. This loss was also identified when corneas were allowed to digest overnight in collagenase or isolated within 4 hours and plated overnight (data not shown), suggesting that the level of ALDH1A1 in particular can fluctuate dramatically depending on environmental conditions. These experiments were repeated more than three times, with each time showing marked reduction of ALDH1A1 and TKT.

The myofibroblast phenotype (Figs. 4A, 4B, lane 3) showed the greatest reduction in expression of both ALDH1A1 (18%) and TKT (27%) compared with *in situ* keratocytes and the other phenotypes representing 2.6% and 5.2% of the total water-soluble protein, respectively. Growth in PDGF (lane 4) produced fibroblasts that expressed comparable levels of ALDH1A1 to serum-free cultured keratocytes (25% or 3.8% of the water-soluble protein) but expressed less TKT (29% of *in situ* keratocytes) only representing 5.5% of the total water-soluble protein. Fibroblasts grown in FGF2 and keratocytes grown in IGF-II showed higher levels of ALDH1A1 and TKT, comparable to levels in serum-free cultured keratocytes, representing 3.7% and 10.0% for FGF2 and 3.8% and 10.0% for IGF-II of the total water-soluble protein, respectively. Overall, the level of expression of these two proteins as a percentage of total water-soluble proteins was markedly reduced under all culture conditions (Fig. 4C), decreasing from 37% of the total for *in situ* keratocytes to 13.0% for serum-free, 7.8% for TGF β -myofibroblasts, 9.3% for PDGF-fibroblasts, 13.7% for FGF2-fibroblasts, and 13.8% for IGF-II keratocytes.

To confirm the phenotypic modulation of keratocytes under the various culture conditions, actin filament organization was evaluated in cells plated on the collagen-coated acrylamide gels and scanned (Fig. 5). Keratocytes cultured in serum-free conditions (Figs. 5A, 5B) or IGFII (Figs. 5C, 5D) showed a characteristic dendritic morphology by *in vivo* confocal reflectance microscopy (Figs. 5A, 5C) with predominantly cortical actin organization after staining with phalloidin (Figs. 5B, 5D). Fibroblastic keratocytes stimulated by PDGF (Figs. 5E, 5F) appeared more elongated and spindle-shaped (Fig. 5E), with more prominent intracellular actin filament bundles (Fig. 5F), whereas FGF2-stimulated fibroblasts appeared

broader and flatter (Fig. 5G) with distinct stress fibers (Fig. 5H). Myofibroblasts cultured with TGF β showed a large, spread morphology (Fig. 5I) with extensive intracellular stress fibers (Fig. 5J). Cells were also cultured on glass coverslips and compared with acrylamide gels and were shown to be essentially identical (data not shown). Furthermore, staining for fibronectin showed little deposited by any phenotype after overnight culture (data not shown).

Scattering of Light by Different Keratocyte Phenotypes

To measure light-scattering from single cells, CMTF data sets were obtained through regions of the collagen-acrylamide gels containing a moderate density of cells. Individual cells were then outlined to define a region of interest within the 3-D data set (Fig. 6A, dotted line), and the pixel intensity was quantified over the region of interest throughout the through-focus series. The average pixel intensity was then plotted as a function of focal plane position for at least three cells within the data set (Fig. 6B). In an average experiment, as shown in Figure 6C, serum-free cultured keratocytes showed the least light-scattering, averaging $574 \pm 66 U_{\text{auc}}$. The level of light-scattering between experiments varied due to changes in the light bulb intensity and camera sensitivity, making it difficult to average levels between experiments. Light-scattering measured from TGF β -myofibroblasts was significantly elevated ($P < 0.05$) to $879 \pm 95 U_{\text{auc}}$, compared with serumfree cultured keratocytes, a consistent finding in at least three separate experiments. Other cell types showed various levels of light-scattering that were always significantly less than in the myofibroblasts, but not significantly different from the serum-free cultured keratocytes. Occasional experiments would detect a significant difference between PDGF-fibroblasts and cultured keratocytes, but this finding was not consistently repeatable.

DISCUSSION

In this study, isolated corneal keratocytes from different species characteristically expressed a few water-soluble enzymes and proteins to levels consistent with that previously considered for inclusion as corneal crystallins obtained from extracts of whole cornea and corneal epithelium.^{1,13,14,29} Furthermore, cultured rabbit keratocytes showed a pattern of expression that varied with the keratocyte phenotype, in that expression was greater in serum-free keratocytes, IGF-II-keratocytes and FGF2-fibroblasts; less in PDGF-fibroblasts; and lowest in TGF β -myofibroblasts. Using a novel method for measuring light-scattering from single cells, significant differences were noted in light-scattering between myofibroblasts and the other cell types. Overall, these findings confirm earlier reports of differences in the expression of keratocyte crystallin proteins from different keratocyte phenotypes in vivo and in vitro,¹⁷⁻¹⁹ and support the findings that wound-healing fibroblasts in vivo scatter light and produce corneal haze and clouding unrelated to the changes in the stromal extracellular matrix.¹²

Recently, the term corneal crystallins has been used to categorize an enlarging group of abundantly expressed water-soluble enzyme and proteins that have been identified in the cornea based on the similarity to abundantly expressed enzymes and proteins in the crystallin lens.^{13,30} In this study, we identified five new abundantly expressed enzymes, including LDH, G3PDH, pyruvate kinase, annexin II, and protein disulfide isomerase (Table 2). Overall, 15 corneal crystallins from diverse species including rabbit, human, bovine, pig, mouse, chicken, fish, and squid have been identified by different investigators.^{12-15,29,31} More important, at least six of the proposed corneal crystallins have lens enzyme crystallin counterparts, as shown in Table 2.³

The function of these abundantly expressed proteins in the cornea however, remains highly speculative. Although an enzymatic function would appear obvious, a metabolic need does not explain the similarity between the expression profiles of the corneal epithelium and keratocytes from the same species, since the biosynthetic and metabolic requirements of the migratory and

proliferative epithelium residing at an aqueous/air interface are undoubtedly quite different from that of the quiescent, sessile keratocyte. Indeed, based on metabolism, it may be more likely that the rabbit keratocyte would show greater similarity to the rabbit scleral fibroblast. Both are quiescent and produce and maintain a collagenous extracellular matrix. Whereas scleral fibroblasts express a similar protein profile, most notably for G3PDH, no protein shows an expression level greater than 3.5% of the total water-soluble protein. In addition, scleral fibroblasts express barely detectable levels of ALDH1A1 and markedly reduced levels of TKT compared with corneal keratocytes, as shown in Western blots (Fig. 2B, lane 2). In light of these considerations, the greater similarity between the corneal epithelium and keratocytes suggests a similar functional role for these abundantly expressed proteins in these two different cell types.

An obvious common feature of both the epithelium and the keratocyte is transparency and the absence of light-scattering in situ. In the present study, significantly increased light-scattering was detected in TGF β -myofibroblasts compared with all other cell types for which the combined expression of TKT and ALDH1A1 were greater. This finding suggests an important link between the abundant expression of these proteins and cellular light-scattering for both the corneal epithelial cells and keratocytes. This relationship is also supported by the finding that PDGF-fibroblasts, which had the next lowest expression level to TKT and ALDH1A1, showed in some experiments significantly greater light-scattering than did cultured keratocytes. Why this was not a consistent finding is not clear, but it should be noted that the overall level of TKT and ALDH1A1 expressed by cultured keratocytes were markedly lower than that of in situ keratocytes, suggesting that cultured keratocyte light-scattering may be elevated, and differences between keratocytes and other phenotypes may not be as great. It should also be noted that light-scattering was greater in IGFII-keratocytes and FGF2-fibroblasts than in cultured keratocytes, and yet they expressed similar levels of TKT and ALDH1A1. This finding suggests that light-scattering may be a complex phenomenon. Further study using this new approach is needed to establish more clearly the overall mechanism of cellular light-scattering.

The level of crystallin protein expression for different keratocyte phenotypes differs from that in a recent report that suggested that PDGF and FGF2, rather than TGF β , were more effective in reducing levels of TKT in cultured corneal keratocytes.³² There are at least two differences in the approach of the present study that explain this discrepancy. First, water-soluble proteins were extracted and analyzed from each cell type, as opposed to the collection of total proteins, which would not discriminate between bound and sequestered proteins and those that are soluble. Second, expression of each crystallin protein was evaluated as a percentage of the total water-soluble proteins, which adjusts for differences in cell volume. As noted in Figure 5, there were marked differences in the cell areas of different keratocyte phenotypes, particularly in TGF β -induced myofibroblasts, which may have twice the cell area or double the cell volume of cultured keratocytes. Comparing differences in expression on a per-cell basis using Western blot analysis does not take into account these differences and would overestimate the abundance of the protein in larger cells such as myofibroblasts. Furthermore, the present in vitro findings are consistent with earlier in vivo observations regarding light-scattering from the rabbit cornea after injury, which show that corneal myofibroblasts scatter markedly greater light than migrating corneal fibroblasts after scrape or laser keratectomy injury.³³

Although it is tempting to propose that the differences in cellular light-scattering are directly related to the more abundant expression of these proteins, it is important to note that expression of TKT and ALDH1A1 is only one of the many characteristics that distinguish rabbit corneal keratocytes from fibroblasts and myofibroblasts. Notable differences include proliferation potential,^{21,28} biosynthesis of extracellular matrix,^{34,35} and development of intracellular actin filament bundles or stress fibers.²² Although conventional wisdom in the past has focused

on the extracellular matrix, in the present study, in which cells were only allowed to attach overnight, the deposition of extracellular matrix should be minimal, and no differences were noted in the deposition of fibronectin. There were major differences in the organization of actin filaments (i.e., stress fibers), between the different phenotypes, and myofibroblasts exhibited the most prominent actin filament bundles. Although FGF2-fibroblasts showed prominent stress fibers and produced significantly less light-scattering than myofibroblasts, no quantitative measurements were performed to show whether actin assembly was significantly different. Certainly, additional evaluation is needed, to establish the underlying mechanism involved in cellular light-scattering by different keratocyte phenotypes. Particularly, the role of actin filaments must be better defined by directly modulating actin filament assembly in the same keratocyte phenotype. Future studies can now be performed using the novel single-cell light-scattering approach that has been developed to answer these questions.

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Artificial Stromal Chamber for Keratocyte Light Scattering Measurements

FIGURE 1.

Diagram of the artificial stromal chamber that was used to measure light-scattering from single cells. Cells were plated on an acrylamide-collagen substrate of 50- μm thickness that was covalently attached to a 2.5-mm diameter glass coverslip. The coverslip was mounted on a baseplate with gasket and then the medium overlaid. A top glass coverslip was then held against the gasket by a locking ring.

FIGURE 2.

(A) SDS-PAGE of water-soluble proteins extracted from the rabbit corneal epithelium (lane 1), keratocytes (lane 2), and corneal endothelium (lane 3). (B) SDS-PAGE and Western blot analysis of ocular tissues, including keratocytes (lane 1), scleral fibroblasts (lane 2), retina (lane 3), and lens (lane 4). Scleral cells showed almost equal loading with corneal keratocytes for G3PDH (IV) yet the levels of expression of TKT and ALDH1A1 were markedly lower. (C) SDS-PAGE and Western blots for TKT, ALDH1A1, and ALDH3A1 of human, mouse, pig, and chicken corneal epithelial cells (lane 1) and keratocytes (lane 2). Major water-soluble proteins identified by protein sequencing: TKT (I), ALDH1A1/3A1 (II), α -enolase (III),

G3PDH (IV), LDH (V), actin (VI), glutathione-S-transferase (VII), pyruvate kinase (VIII), protein disulfide isomerase (IX), and annexin II (X).

FIGURE 3.

Light-scattering from phantom corneas containing a 150-μm thick-central formazin gel. **(A)** Confocal reflectance image through a 9000-NTU formazin gel showing clumping of formazin particles within the gel. **(B)** CMTF reconstruction of the phantom cornea with 9000 NTU formazin gel. *Arrow:* light-scattering from the glass coverslip. **(C)** Depthintensity profile taken from the CMTF data shown in **(B)**. *Arrow:* light-scattering from the glass coverslip. **(D)** Average light-scattering from phantom corneas with different formazin gel concentrations. Bar, 100 μm.

FIGURE 4.

Expression of water-soluble proteins from different keratocyte phenotypes, including in situ keratocytes (*lane 1*), serum free (*lane 2*), TGF β -myofibroblasts (*lane 3*), PDGF-fibroblasts (*lane 4*), FGF2-fibroblasts (*lane 5*), and IGF-II-keratocytes (*lane 6*). (A) SDS-PAGE. (B) Western blot for TKT and ALDH1A1. (C) Graph of TKT and ALDH1A1 expressed as the percentage of total water-soluble protein.

FIGURE 5.

Confocal reflectance (**A, C, E, G, I**) and fluorescence (**B, D, F, H, J**) microscopy of keratocyte phenotypes on collagen-coated gels stained with FITC-phalloidin (*green*) and propidium iodide (*red*). (**A, B**) serum-free keratocytes, (**C, D**) IGF-II-keratocytes, (**E, F**) PDGF-fibroblasts, (**G, H**) FGF2-fibroblasts, (**I, J**) TGF β -myofibroblasts. Bar, 100 μ m.

FIGURE 6.

Light-scattering measurements from single cells. (A) Confocal reflectance micrograph of PDGF cells on collagen coated acrylamide gels. *Dotted line*: cell outlined as a region of interest. (B) Measurement of average pixel intensity from different focal planes through three different cells from the CMTF data set shown in (A). (C) Graph of average light-scattering measurements for different keratocyte phenotypes. *Significant difference between TGF β -myofibroblasts and serum free keratocytes; **Significant difference between TGF β -myofibroblasts but no difference between serumfree keratocytes. Bar, 100 μm .

TABLE 1

Abundantly Expressed Proteins in Rabbit, Human, Mouse, Pig, and Chick

Abundant Protein	MW	Rabbit		Human		Mouse		Pig		Chick	
		Ker	Epi	Ker	Epi	Ker	Epi	Ker	Epi	Ker	Epi
I Transketolase	73	14.0	7.5	6.8	13.7	4.3	8.8	11.5		11.5	5.9
II ALDH 1A1/3A1	54	12.7	4.3	14.7	11.8	5.3	12.8		33.0		
III α -Enolase	47		4.5	9.5	7.0			4.5		4.5	3.5
IV G3PDH	36	3.8	6.9	5.7	7.1			3.5		3.5	7.5
V LDH	36	3.8	14.7	3.5				8.0		5.4	5.9
VI Actin	43						4.0				
VII Glutathione S-transferase	27						4.2				
VIII Pyruvate kinase	58							4.7		4.7	5.0
IX Protein disulfide isomerase	55							4.5		4.5	7.4
X Annexin II	39							6.2		6.2	6.2

Roman numerals coincide with those marking the bands in Figure 2. Ker, keratocyte; epi, epithelium.

TABLE 2
Abundantly Expressed Corneal Proteins and Lens Enzyme Crystallins

Corneal Crystallin	Species	Ref.	Lens Crystallin	Species
ALDH1A1	rabbit, human, pig	12	η-crystallin	Elephant shrew
α-Enolase	human, mouse, chicken	13	τ-crystallin	Lamprey
Glutathione S-transferase	squid, mouse	13	Ω-crystallin	Cephalopod
LDH	rabbit, human, chicken, pig	*	ε-crystallin	Duck, crocodile
G3PDH	rabbit, human, chicken	*	π-crystallin	Gecko
Argininosuccinate lyase	chicken	13	δ-crystallin	Birds, reptile
TKT	mammals	12, 29		
BCP54/ALDH3A1	most mammals	31		
Isocitrate dehydrogenase	bovine	14		
Gelsolin	fish	15, 16		
Actin	fish, mouse	15, 16*		
Peptidyl-prolyl cistrans isomerase	chicken	13		
Pyruvate kinase	chicken	*		
Annexin II	chicken	*		
Protein disulfide isomerase	chicken	*		

* Current report.