Myofibroblast Differentiation Modulates Keratocyte Crystallin Protein Expression, Concentration, and Cellular Light Scattering

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PURPOSE. The purpose of this study was to determine whether myofibroblast differentiation altered keratocyte crystallin protein concentration and increased cellular light scattering.

METHODS. Serum-free cultured rabbit corneal keratocytes and TGF β (5 ng/mL) induced myofibroblasts were harvested and counted and protein/RNA extracted. Expression of myofibroblast and keratocyte markers was determined by real-time PCR and Western blot analysis. The cell volume of calcein AM-loaded keratocytes and myofibroblasts was determined by using nonlinear optical microscopy. Cellular light scattering of transformed myofibroblasts expressing human keratocyte crystallins was measured by reflectance confocal microscopy.

RESULTS. Differentiated myofibroblasts showed a significant decrease in RNA levels for the keratocyte markers ALDH1A1, lumican, and keratocan and a significant increase in the myofibroblast marker α -smooth muscle actin. Volumetric and protein measurements showed that myofibroblast differentiation significantly increased cytoplasmic volume (293%; $P < 0.001$) and water-soluble and -insoluble protein content per cell (respectively, 442% and 431%; $P < 0.002$) compared to keratocytes. Western blot analysis showed that the level of ALDH1A1 protein per cell was similar between myofibroblasts and keratocytes, but was substantially reduced as a percentage of total water-soluble protein. Light scattering measurements showed that induced expression of corneal crystallins significantly decreased light scattering.

CONCLUSIONS. These data suggest that myofibroblast differentiation leads to a marked increase in cell volume and dilution of corneal crystallins associated with an increase in cellular light scattering. (*Invest Ophthalmol Vis Sci.* 2012;53:770–778) DOI: 10.1167/iops.11-9092

Previous studies have shown that differentiation of keratocytes into myofibroblasts is regulated in part by transforming growth factor (TGF)- β .^{1,2} Under serum-free culture condi-

tions, rabbit, bovine, and human corneal keratocytes maintain a quiescent phenotype, dendritic morphology, and high expression of phenotypic markers, including keratocan, lumican, and the corneal crystallins, aldehyde dehydrogenase isozymes 3A1 and 1A1 (ALDH3A1/1A1) and transketolase (TKT).^{3–5} Treatment of cultured keratocytes with TGF β leads to cell spreading, actin filament assembly, and downregulated expression of keratocyte-specific genes that is coupled with the de novo upregulated expression of α -smooth muscle actin (α -SMA), the phenotypic marker for myofibroblast differentiation.⁶ Myofibroblasts play a critical role in corneal wound healing that involves deposition and organization of extracellular matrix leading to wound contraction.^{7–9} Neutralizing antibodies to TGF β have been shown to block the appearance of myofibroblasts in corneal wounds and significantly reduce corneal scarring and the development of corneal haze.^{10,11}

The loss of transparency after corneal injury and scarring has long been associated with the deposition of abnormally arranged and disorganized scar collagen. Studies have shown that in regions of scarring, collagen fibrils show a more disordered structure with greater variation in collagen fibril size and spacing that is typically associated with prolonged, if not permanent, loss of corneal transparency.^{12–14} With the development of excimer laser photorefractive keratectomy (PRK), temporary loss of corneal transparency has been noted as corneal haze, which, in patients, typically peaks 3 months after PRK surgery and resolves by the end of the first year, although a late-onset corneal haze has been reported that is treated by retreatment with topical steroids.¹⁵ Topical steroids reduce corneal inflammation, but also suppress the release of TGF β ¹⁶ and have been shown to reduce the appearance of corneal myofibroblasts and corneal haze after anterior lamellar keratectomy in rabbits.¹⁷ Although corneal myofibroblasts deposit abnormal extracellular matrix, these transient changes in corneal haze that are observed after PRK are not easily explained by the process of extracellular matrix remodeling in response to topical steroids.

As an alternative explanation, a cellular basis for corneal transparency has been proposed based on the expression of keratocyte-specific corneal crystallins.¹⁸ Corneal crystallins represent a diverse group of enzymes/proteins that are abundantly expressed by all corneal cells, exceeding more than 50% of the total water-soluble cytoplasmic protein in some species.^{19–22} Since the expression of these proteins is similar to that of the lens, both in abundance and taxon specificity, it has been suggested that corneal crystallins play a role in determining the transparent and refractive properties of the cornea through a metabolic or structural function.²³ In support of this hypothesis, expression of corneal crystallins has been shown to be environmentally and developmentally regulated with a marked increase in expression after eyelid opening, exposure to light, and development of corneal transparency.^{24,25}

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Studies of corneal injury and wound repair in both humans and laboratory animals have shown that wound-healing fibroblasts and myofibroblasts have significantly reduced expression of corneal crystallin proteins.^{18,26} Furthermore, wound-healing fibroblasts and myofibroblasts show a marked increase in light scattering or corneal haze, as detected by in vivo confocal reflectance microscopy.^{10,27,28} Growth factor-stimulated rabbit keratocytes in culture also show significantly reduced levels of crystallin protein and increased cellular light scattering, with TGF β -induced myofibroblasts showing the greatest changes.²²

Although expression of corneal crystallins has been shown to correlate with cellular light scattering and corneal haze, the molecular and biophysical mechanisms affecting cellular transparency have yet to be explained. In this study, we evaluated the biophysical characteristics of keratocytes and myofibroblasts on the basis of cell size, protein content, expression of corneal crystallins, and changes in light scattering after induced corneal crystallin expression. Our findings suggest that during myofibroblast differentiation, keratocyte cell volume and protein content dramatically increase, leading to a marked dilution of corneal crystallin content. Furthermore, induced expression of corneal crystallins appears to significantly decrease light scattering in a transformed rabbit corneal myofibroblast cell line. Taken together, these findings suggest that keratocyte crystallin proteins have concentration-dependent effects on light scattering from cells, which may involve structural or enzymatic functions or both.

METHODS

Rabbit Keratocyte and Myofibroblast Culture

Keratocytes were isolated from rabbit eyes (Pel-Freez, Rogers, AR) according to previously described techniques.¹ The cells were initially plated in DMEM (Invitrogen Corp, Carlsbad, CA) containing 1% RPMI vitamin mix (Sigma-Aldrich, St. Louis, MO), nonessential amino acids (Invitrogen Corp.), 289 μ g/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich), and 1% penicillin-streptomycin-amphotericin B (Invitrogen Corp.) in tissue culture dishes (Primaria; BD Biosciences Labware, Franklin Lakes, NJ) or bovine-collagen-coated (PureCol; Advanced BioMatrix, San Diego, CA) glass coverslips at a density of 1.0×10^4 cells/cm² and cultured at 37°C in a humidified 5% CO₂ incubator for all experiments. The cells were allowed to attach for 3 to 4 days before the addition of TGF β 1 (5 ng/mL, Sigma-Aldrich) to induce myofibroblast differentiation. They were then cultured for 3 days to allow myofibroblast differentiation and then were used to determine RNA expression, cell fractionation, protein levels, and cell volume and for immunocytochemistry. Proteins isolated from the cells were also evaluated by Western blot analysis, according to published methods.²²

Real-Time PCR Evaluation of Keratocyte and Myofibroblast Markers

Cells were lysed using RLT buffer and the RNA isolated over spin columns using the manufacturers protocol (RNeasy; Qiagen, Valencia,

CA). RNA yield was determined with a spectrophotometer (Nanodrop; Thermo Scientific, Wilmington, DE). Gene expression was then evaluated by real-time PCR, according to published methods.^{29,30} Briefly, 0.5 μ g RNA was reverse transcribed using oligo dT and random primers as supplied in the reverse-transcription kit (QuantiTect; Qiagen). Real-time PCR was performed using SYBR green reagents (Power SYBR Green; Applied Biosystems, Inc. [ABI], Foster City, CA) and validated real-time PCR primers for rabbit keratocyte and myofibroblast markers, as listed in Table 1. Products were evaluated by melt curve analysis, sizing on agarose gels, and sequencing. Relative quantification was performed by the $\Delta\Delta C_T$ method, with both GAPDH and β -actin used as the normalizing housekeeper genes, as previously described.²⁹

Extraction and Measurement of Water-Soluble and -Insoluble Cell Protein

Keratocytes and myofibroblasts were collected from tissue culture plates by trypsinization, washed in phosphate-buffered saline (pH 7.4), counted (Vi-Cell Cell Viability Analyzer; Beckman Coulter Inc., Miami, FL), and suspended in extraction buffer containing 25 mM Tris-HCl (pH 7.4), with 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 μ g/mL antipain, 5 μ g/mL pepstatin A, and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). The cells were sonicated at 4°C for less than 1 minute and stored for 1 hour on ice to extract the water-soluble proteins. The samples were centrifuged at 12,000g for 3 minutes and the supernatant collected. The pellet was resuspended in extraction buffer containing 0.5% SDS and the proteins boiled for 10 minutes to collect insoluble proteins. The amount of protein was determined by a DC protein assay modified for use with thiols (Bio-Rad Laboratories, Hercules, CA). For each experiment, two 10-cm dishes of cells were separately analyzed, and the protein concentration per cell was determined for each plate. Each experiment was run three separate times, and the average of the six plates is reported.

Measurement of Keratocyte and Myofibroblast Cell Volume

Keratocytes and myofibroblasts plated on coverslips were initially loaded with the fluorescent probe calcein, by incubating cells in culture medium containing 2 μ M calcein AM (Invitrogen Corp.) at 37°C for 30 minutes. Cell nuclei were also labeled by co-incubation with 3.3 μ g/mL DAPI (Invitrogen Corp.). After loading, the coverslips were transferred to an incubator chamber (Incubator S, attached to a CTI-Controller 3700; Carl Zeiss Meditec, Jena, Germany), to maintain humidity, in 5% CO₂. The cells were then imaged by nonlinear optical microscopy (510 Meta LSM; Carl Zeiss Meditec, Inc., Thornwood, NY) using a 150-fs titanium sapphire laser (Chameleon; Coherent, Santa Clara, CA) tuned to 800 nm to excite calcein and DAPI. Calcein and DAPI emission spectra were then simultaneously collected with an NFT490 dichroic mirror combined with BP390-465 and BP500-550 IR bandpass filters.

To quantify cell volume, 3-D data stacks were collected of individual cells using the 40 $\times$ /1.2 NA water-immersion objective (Apochromat; Carl Zeiss Meditec) with a voxel resolution of $0.44 \times 0.44 \times 0.5$ μ m (x, y, z). All data stacks were collected using the same laser power and detector gain settings for each experiment. Image data sets were

TABLE 1. Rabbit Primers Used for RT-PCR

Gene	Accession No.	Primer	Primer	Expected Size (bp)	Sequenced
<i>Lumican</i>	AF020292	TGCAGCTTACCCACAACAAG	AGGCAGTTTGCTCATCTGGT	176	YES
<i>Tgfb2</i>	AY429466.1	GACCCACATCTCTGCTAA	CACCCAAGATCCCTCTTGAA	165	YES
<i>Tgfb1</i>	AF000133.1	TGCTTCAGCTCCACAGAGAA	CTTGCTGTACTGGGTGTCCA	162	YES
<i>Keratocan</i>	DQ239829.1	GTCTCACAATCGCCTCAGAA	GGTCCATGGATGAACGAATC	153	YES
<i>Aldb1a1</i>	AY038801.1	ACTCCCTCACTGCTCTTCA	AACACTGGCCCTGATGGTAG	316	YES
<i>Sma</i>	X60732.1	TGCTGTCCCTCTATGCCTCT	GAAGGAATAGCCACGCTCAG	148	YES
<i>Actin</i>	AF309819.1	ATCGTGATGGACTCCGGGAGC	AGCGCCACGTAGCAGACG	211	YES
<i>Gapdh</i>	L23961.1	GAGCTGAACGGGAACTCAC	CCCTGTTGCTGTAGCCAAAT	304	YES

then transferred to a workstation running image-processing software (Metamorph; Molecular Devices, Downingtown, PA). Image stacks were then thresholded using the same thresholds for each experiment, to identify positive fluorescence, and the pixel area was quantified by using the measuring subroutine for each plane. The data were then transferred to a spreadsheet (Excel; Microsoft, Redmond WA) and the total cell and nuclear volume determined by summing across all planes. To determine cytoplasmic volume, the nuclear volume was subtracted from the total cellular volume. Two coverslips for each cell type were evaluated for each experiment, and the experiment was repeated three times. The average cytoplasmic and nuclear volumes for all cells evaluated in the three different experiments are reported.

ALDH Transfection and Measurement of Single-Cell Light Scattering

An SV40-transformed rabbit corneal myofibroblast cell line that has been shown to have constitutive expression of α -SMA was used³¹ and stably transfected with human ALDH3A1 or ALDH1A1, as previously described.³² Single-cell light scattering was measured by using previously reported methods.²² Briefly, the cells were plated onto 25-mm diameter glass coverslips containing a 10-mm diameter, 50- μ m-thick acrylamide gel/collagen substrate. Coverslips were placed in the base ring of the artificial stromal chamber and mounted on a heat block to maintain temperature at 37°C. Through-focusing data sets were collected with a confocal microscope (Tandem Scanning Corp., Reston, VA). At least three CMTF data sets covering three to four cells were taken from three different regions for each coverslip and two coverslips for each cell type were evaluated for each experiment. To measure light scattering from single cells, 3-D data sets were transferred to a workstation (MetaMorph; Molecular Devices), and the cellular light scattering was measured as previously reported.²² This experiment was repeated three times, and the average single-cell light scattering for each cell type for each experiment was reported.

Immunostaining of Keratocytes and Myofibroblasts

Keratocytes, TGF β -induced myofibroblasts, and a SV40-transformed rabbit corneal myofibroblast cell line were immunostained with antibodies against ALDH3A1 and ALDH1A1, as previously described.^{32,33} Briefly, the cells were grown on collagen-coated glass coverslips and then fixed in 2% paraformaldehyde in phosphate-buffered saline (pH 7.4). They were permeabilized and reacted with rabbit anti-human ALDH3A1 or with chicken anti-rabbit ALDH1A1 (both diluted 1:100 in PBS). The samples were washed, stained with FITC-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-chicken IgY, and counterstained with 4',6-diamidino-2-phenylindole (DAPI), to identify the nucleus. The cells were then evaluated by microscope with a 40 $\times$ objective (LSM 510 Meta; Carl Zeiss Meditec).

Statistical Analysis

All results are reported as the mean $\pm$ SD. Differences between groups were assessed by one-way ANOVA and Bonferroni multiple comparisons (Sigma Stat ver. 3.11; Systat Software Inc., Point Richmond, CA).

RESULTS

Effects on Gene Expression, Cell Volume, and Protein Concentration

Since GAPDH has been reported to be a corneal crystallin in rabbits,²² gene expression levels for keratocyte and myofibroblast markers in tissue-cultured keratocytes and TGF β -induced myofibroblasts were compared by normalization to β -actin (Table 2). Based on two-way ANOVA of the $\Delta\Delta C_T$ values, TGF β -induced myofibroblast differentiation significantly reduced expression of keratocyte-specific genes, including ALDH1A1 ($P < 0.05$), lumican ($P < 0.01$), and keratocan ($P < 0.05$).

TABLE 2. Expression of Keratocyte versus Myofibroblast Genes

Transcript	C_T RK	C_T MYO	ΔC_T MYO*	$\Delta\Delta C_T \dagger \Delta C_T - \Delta C_T$ RK	$\Delta\Delta C_T \dagger \Delta C_T - \Delta C_T$ MYO	Relative Level $\ddagger$ RK	Relative Level $\ddagger$ MYO
GAPDH	21.85 $\pm$ 0.93	23.57 $\pm$ 0.75	1.37 $\pm$ 0.76	0.00 $\pm$ 1.69	0.56 $\pm$ 0.76	1.00 (0.31–3.22)	0.68 (0.40–1.15)
β -Actin	21.04 $\pm$ 1.41	22.20 $\pm$ 0.10	0.00 $\pm$ 0.14	0.00 $\pm$ 1.99	0.00 $\pm$ 0.14	1.00 (0.25–3.97)	1.00 (0.91–1.11)
TGF β 2	22.53 $\pm$ 0.57	26.42 $\pm$ 0.11	4.22 $\pm$ 0.15	0.00 $\pm$ 1.52	2.73 $\pm$ 0.15	1.00 (0.35–2.86)	0.15 (0.14–0.17)
TGF β 1	24.70 $\pm$ 0.67	25.35 $\pm$ 0.60	3.15 $\pm$ 0.60	0.00 $\pm$ 1.56	–0.51 $\pm$ 0.60	1.00 (0.34–2.95)	1.43 (0.94–2.17)
α SMA	27.02 $\pm$ 0.99	24.33 $\pm$ 0.31	5.98 $\pm$ 1.72	0.00 $\pm$ 1.72	–3.86 $\pm$ 0.32§	1.00 (0.30–3.30)	14.53 (11.60–18.19)
Aldh1a1	22.75 $\pm$ 1.06	27.09 $\pm$ 0.34	4.88 $\pm$ 0.36	0.00 $\pm$ 1.76	3.17 $\pm$ 0.36	1.00 (0.29–3.40)	0.11 (0.09–0.14)
Lumican	16.52 $\pm$ 0.69	20.66 $\pm$ 0.63	–4.52 $\pm$ 1.57	0.00 $\pm$ 1.57	2.98 $\pm$ 0.64	1.00 (0.34–2.97)	0.13 (0.08–0.20)
Keratocan	16.96 $\pm$ 1.07	22.31 $\pm$ 0.73	–4.08 $\pm$ 1.77	0.00 $\pm$ 1.77	4.19 $\pm$ 0.74§	1.00 (0.29–3.41)	0.05 (0.03–0.09)

* ΔC_T was determined by subtracting the transcript of interest C_T from the β -Actin C_T . The standard deviation of the difference is calculated from the standard deviations of the transcript of interest and β actin values.

† $\Delta\Delta C_T$ was calculated by subtracting the ΔC_T of the rabbit keratocyte from the ΔC_T of the sample. As this was an arbitrary constant, the standard deviation is equivalent to the standard deviation of the ΔC_T .

‡ The range for the relative transcript level was determined by the expression: $2^{-(\Delta\Delta C_T \pm SD)}$.

§ $P < 0.01$ based on two-way ANOVA of $\Delta\Delta C_T$ values.

|| $P < 0.05$.

FIGURE 1. Calcein AM-loaded (green) and DAPI-stained (blue) live keratocytes (A, a) and myofibroblasts (B, b) shown in maximum-intensity projections along the *x-y* (A, B) and *x-z* (a, b) planes. Note that the keratocytes appear as flattened dendritic cells compared with the highly spread and swollen myofibroblasts.

0.05) resulting in a respective 89%, 87%, and 95% loss in expression levels. In contrast, the expression level of α -SMA in myofibroblasts significantly increased ($>1400\%$; $P < 0.01$) compared with the level expressed in the keratocytes. It should also be noted that treatment of keratocytes with TGF β 1 appeared to lead to upregulation of TGF β 1 expression by 43% and downregulation of TGF β 2 expression by 95%, although these differences were not significant in this study.

To assess changes in cell volume induced by treating keratocytes with TGF β 1 for 3 days, cells were loaded with the intracellular fluorescent dye, calcein AM, counterstained with DAPI to identify the cell nucleus, and then three-dimensionally imaged using nonlinear optical microscopy. Qualitatively, keratocytes showed a dendritic morphology with an apparent high nuclear-to-cytoplasmic ratio (Fig. 1A) compared with the polygonal shaped and highly spread morphology of myofibroblasts (Fig. 1B). Keratocytes also appeared to be considerably flatter than myofibroblast when viewed in *x-z* projections (Figs. 1A, 1B, respectively). As shown in Table 3, quantitation of myofibroblasts cellular volume based on three-dimensional image data showed that there was a significant increase ($P < 0.001$) in the intracellular volume excluding the nucleus from $4,250 \pm 1,340 \mu\text{m}^3$ on average in keratocytes to $12,460 \pm 5,590 \mu\text{m}^3$ on average in myofibroblasts, for a 293% increase

(Table 3). The nuclear volume of myofibroblasts was also significantly increased ($P < 0.001$); however, the nuclear-to-cytoplasmic ratio was significantly lower (64%; $P < 0.001$) compared with that in the keratocytes.

To assess the amount of cellular protein, cells were trypsinized, counted, and fractionated into water-soluble and -insoluble fractions. Measurement of protein levels adjusted for the number of cells showed that myofibroblasts contained significantly greater ($P < 0.002$) water-soluble and -insoluble protein on a per cell basis, averaging 442% and 431% higher compared with keratocytes (Table 3). However, the ratio of water-soluble to -insoluble protein per cell was not significantly different between myofibroblasts and keratocytes. Although it was not possible to directly measure the amount of protein and cell volume in the same cells, calculation of protein/cell volume from the different group measurements suggested that myofibroblasts contained approximately 151% and 147% more water-soluble and -insoluble proteins per unit cell volume than did the keratocytes.

Changes in ALDH1A1

To understand how changes in cell volume and protein expression influences corneal crystallin protein levels, keratocytes

TABLE 3. Keratoocyte versus Myofibroblast Protein and Cell Volume

Cell Fraction	Keratoocyte			Myofibroblasts			% Change	<i>P</i>
	<i>n</i>	Mean	SD	<i>n</i>	Mean	SD		
Volume ($\times 10^3 \mu\text{m}^3$)	43†			31				
Cytoplasmic		4.25	1.34		12.46	5.58	293	<0.001
Nuclear		0.55	0.13		0.90	0.27	164	<0.001
Ratio		0.14	0.04		0.09	0.06	64	<0.001
Protein (pg/cell)	6†			6				
Water soluble		22.4	5.3		98.8	25.0	442	<0.002
Insoluble		68.2	14.8		294.3	214.8	431	<0.002
Ratio		0.34	0.10		0.5	0.32	152	NS
Protein (pg/cell volume ($\times 10^3 \mu\text{m}^3$))								
Water soluble		5.3			7.9		151	
Insoluble		16.1			23.6		147	

* Kruskal-Wallis one-way analysis of variance on ranks.
† Number of individual cells measured.
‡ Number of individual plates analyzed.

FIGURE 2. ALDH1A1 (green) immunostaining of keratocytes (A, B) and myofibroblasts (C, D) shown in a single x - y plane (A–D) and an x - z plane (a–d). The keratocytes showed more intense staining compared with myofibroblasts, and ALDH1A1 accumulated in the nucleus of keratocytes (A, a, arrow) but not in the nucleus of myofibroblasts (B, b, arrow). This is confirmed based on co-localization of ALDH1A1 staining with nuclear DAPI staining (B and b and D and d, respectively).

and myofibroblasts were initially immunostained with antibodies to ALDH1A1. As shown in Figure 2, the keratocytes showed more intense immunostaining for ALDH1A1 in the cell body and dendritic cell processes (Figs. 2A, 2B) than did the myofibroblasts (Figs. 2C, 2D). In addition, the keratocytes showed ALDH1A1 immunostaining localized to the nucleus (Figs. 2A, 2a, arrow), whereas little or no nuclear staining was detected in the myofibroblasts (Fig. 2C, 2c, arrow). To confirm differences in ALDH1A1 expression, total protein extracts from keratocytes and myofibroblasts were subjected to Western blot analysis (Fig. 3). When equal total proteins were loaded on each lane, there was a substantial reduction in the amount of ALDH1A1 within myofibroblasts (Myo) compared with keratocyte (RK) protein extracts. However, if an equal number of cells was loaded ($\times 10^6$ cells/lane), then approximately equal levels of ALDH1A1 were detected in keratocytes and myofibroblasts. Overall, the data suggest that there was a substantial dilution of ALDH1A1 within myofibroblasts, with little degradation during the 3 days of myofibroblast differentiation.

Effects of Induced ALDH3A1/1A1 Expression on Cell Light Scattering

To evaluate the effects of corneal crystallin expression on cellular light scattering, an SV40-transformed rabbit corneal keratocyte cell line (TRK), which has been shown to constitutively express α SMA,³¹ was transfected with expression vectors for human ALDH3A1 or ALDH1A1. Clones were then selected and evaluated for expression levels of ALDH3A1/1A1

by Western blot analysis. As shown in Figure 4, two clones transfected with vectors showed high expression of human ALDH3A1 (lanes 6 and 7; clones 17 and 6, respectively). One clone transfected with human ALDH1A1 (lane 4; clone 20) showed elevated expression, whereas a second clone showed little or no expression (lane 5; clone 21). It should be noted that human ALDH1A1 protein appeared to have a slightly

FIGURE 3. Western blots of total cellular proteins extracted from keratocytes (RK) and myofibroblasts (Myo). Proteins were run based on equivalent loading (10 μ g/lane) or equivalent number of cells (10^6 cells/lane). The myofibroblasts showed a substantial reduction in ALDH1A1 based on equal protein load, with little change based on equal number of cells.

FIGURE 4. Western blot analysis of cellular proteins obtained from normal rabbit keratocytes (*lane 1*), SV40 transformed rabbit keratocytes (TRK, *lane 2*), empty vector transfected TRK (*lane 3*), ALDH3A1 vector transfected TRK (*lanes 6 and 7*; clones 17 and 6), and ALDH1A1 vector transfected TRK (*lanes 4 and 5*; clones 20 and 21). Note that TRK show no expression of ALDH3A1 and low expression of ALDH1A1 compared to normal keratocytes. Furthermore, the molecular weight of human ALDH1A1 appears to be slightly greater than that of rabbit ALDH1A1.

higher molecular weight than did the endogenous rabbit protein. Furthermore, rabbit keratocytes lack expression of ALDH3A1.¹⁸

To confirm expression patterns, transfected cells were immunostained with antibodies to ALDH3A1 (Fig. 5). Although transfected cells maintained a spread myofibroblast morphology, cells showed intense immunostaining throughout the cell cytoplasm, similar to that detected in normal rabbit keratocytes. However, clone 6 (Figs. 5A, 5B) showed nuclear localization of ALDH3A1 (arrow), whereas clone 17 (Figs. 5C, 5D) showed no nuclear ALDH3A1 (arrow). Immunostaining of the clone 20 with expression of ALDH1A1, showed a similar intense staining pattern with nuclear co-localization to that of clone 6 (data not shown).

Individual clones were then plated onto acrylamide/collagen-coated substrates and evaluated for cellular light scattering in an artificial corneal chamber. In each experiment, light-scattering measurements were obtained from all four clones on the same day, and the experiment was repeated three times. As shown in Figure 6, light scattering from clone 20 (1A1 C20) and clone 6 (3A1 C6) were significantly lower ($P < 0.026$ and $P < 0.006$, respectively) than the light scattering measured from the empty vector clone (pCEP4) based on statistical evaluation of the average of all three independent experiments. Although light scattering from clone 17 (3A1 C17) was less than that of the empty vector clone and although individual experiments 1 and 3 showed significantly lower light scattering, the data from the three independent experiments was not significantly different.

FIGURE 5. Immunostaining for ALDH3A1 (green) of clone 6 (A, B) and clone 17 (C, D) transfected with the ALDH3A1 expression vector. Both clones showed intense cytoplasmic ALDH3A1 staining. However, clone 6 also showed nuclear localization of ALDH3A1 (A and a, arrow) that was not observed in clone 17 (B and b, arrow). The nuclear localization was also confirmed by co-localization with DAPI (blue; B and D), and in x-z optical sections through the nuclear region (a-d).

FIGURE 6. Single-cell light scattering measurement taken from control empty vector clone (pCEP4), clone 20 (1A1 C20), clone 6 (3A1 C6), and clone 17 (3A1 C17). In all three experiments, clone 20 and clone 6 showed significantly lower values compared with the empty vector clone. Clone 17 showed significantly lower light scattering in experiments 1 and 3. Statistical analysis on all three experiments showed significant differences for clone 20 and clone 6.

DISCUSSION

This article establishes for the first time that TGF β -induced myofibroblast differentiation in culture leads to significant changes in rabbit corneal keratocyte cell volume and total protein expression resulting in a 293% increase in cell size and a 442% increase in cellular protein on a per cell basis. The increase in cell size and protein content was also accompanied by a significant decrease in RNA expression for the corneal crystallin, ALDH1A1, as well as other keratocyte-specific genes. Western blot analysis of protein extracts based on equal protein load compared with an equal number of cells indicated that the amount of ALDH1A1 on a per cell basis was not changed, whereas the contribution to total protein was substantially decreased. Taken together, these findings establish that increased cell size and protein synthesis during myofibroblasts differentiation leads to the dilution of corneal crystallins within keratocytes. Our finding that enhanced expression of human ALDH1A1 and 3A1 after transfection of a myofibroblast cell line results in decreased cellular light scattering further suggests that the dilution of corneal crystallins may explain in part increased cellular light scattering of myofibroblasts and the development of corneal haze after refractive surgery.

Although past studies have noted that myofibroblast differentiation involves a dramatic change from a dendritic to a spread morphology,^{1,34} the effect of this change on cell size and protein content has not been measured. The expression of corneal crystallins in keratocytes has been studied and shown to be substantially decreased by culture in serum- or growth factor-supplemented medium.^{22,26,35–37} Studies of TKT, an abundantly expressed mammalian corneal crystallin, have also established that the transition of keratocytes to serum-cultured, repair fibroblasts involves degradation of TKT by the ubiquitin-proteasome pathway.³⁶ However, there has been conflicting evidence regarding the expression of TKT in cultured myofibroblast, with one report suggesting that corneal crystallin protein expression is uncoupled from TGF β -induced myofibroblast differentiation.³⁷ Our current results support and extend these earlier findings and indicate that corneal crystallins are not substantially degraded during early myofibroblast differentiation. Nevertheless, the loss of ALDH1A1 expression coupled

with the dramatic increase in cell volume and protein content indicates that the cellular concentration of corneal crystallins can be dramatically altered without ubiquitin-proteasome degradation. Furthermore, these and earlier findings showing increased/decreased cellular light scattering in cells with decreased/increased corneal crystallin protein content strongly suggest that corneal crystallins have concentration-dependent effects on corneal transparency. This concentration dependency may provide important clues to the cellular and biophysical mechanisms relating cellular transparency to corneal crystallin protein expression.

ALDH1A1 and ALDH3A1 are mammalian corneal crystallins, commonly expressed in a taxon-specific pattern, which can comprise greater than 50% of the total water-soluble protein within corneal keratocytes.²² ALDH3A1/1A1 are members of a superfamily of NAD(P)⁺-dependent enzymes that are involved in the metabolism of highly toxic and long-lived lipid peroxides and aldehydes, including 4-hydroxy-2-nonenal and malondialdehyde.³⁸ These enzymes are thought to play a critical role in the protection of the cornea and lens to UV-induced damage through multiple mechanisms including absorption of UV light,³⁹ metabolism of toxic aldehydes,^{40,41} direct free radical scavenging,^{32,42} and production of antioxidant NAD(P)H.³³ The sum of these actions may help block oxidative stress-induced protein modifications leading to enzyme inactivation, partial unfolding, and non-native protein aggregation.

Oxidative stress and protein modification are thought to play important roles in the formation of cataract,⁴³ suggesting that a similar mechanism is involved in the development of corneal haze and increased light scattering after corneal injury and repair. In support of this hypothesis, we have shown that stably transfected myofibroblast cell lines expressing ALDH3A1 protect against oxidative damage due to a range of oxidants, including H₂O₂, mitomycin C, and etoposide.³² Our finding that myofibroblasts with induced ALDH3A1/1A1 expression show significantly reduced cellular light scattering is also consistent with protection against oxidative stress leading to protein aggregation. It should be noted that although the induced expression is significantly above that detected in the parental cell clone, the level of crystallin protein expression remains substantially below that of native keratocytes, suggesting perhaps an enzymatic rather than structural role of corneal crystallins. Similarly, recent findings⁴⁴ that mice deficient in ALDH3A1 and ALDH1A1 show increased development of cataract and increased corneal and lens sensitivity to UV damage leading to greater corneal edema and haze provide additional support for corneal crystallins playing an antioxidant/enzymatic role in the maintenance of corneal transparency through inhibiting protein aggregation.

Alternatively, the effects of ALDH1A1 on light scattering may be associated with other structural mechanisms involving protein-protein interactions. ALDH1A1 is a multifunctional protein and protein-protein interactions as well as protein-small molecule interactions may be expected in the keratocytes as it occurs in other cell types.⁴⁵ As discussed by Møller-Pederson,²⁷ dipolar electrical properties of proteins may influence the cytoplasmic structure leading to reduced large particle light scattering. Such biophysical mechanisms may be analogous to the effects of collagen fibril size and spacing, influencing light scattering from the extracellular matrix in the cornea. Certainly, additional studies are needed to more clearly understand the role of ALDH3A1/1A1 in keratocyte cell function.

An additional finding in this study was the observation that the nuclear localization of ALDH1A1 is lost during TGF β -induced myofibroblast differentiation. Although the function of nuclear ALDH is unknown, previous studies in human corneal epithelial cells lines have shown that stable transfection

of ALDH3A1 leads to elongation of the cell cycle, suggesting that ALDH3A1 may serve as a negative cell cycle regulator.⁴¹ That ALDH1A1 may serve a similar nuclear function is supported by the fact that keratocytes are quiescent and do not enter the cell cycle, whereas myofibroblasts are proliferative. The two myofibroblast cell lines stably transfected with ALDH3A1 showed either nuclear localization or nuclear exclusion. Since the parental myofibroblast cell line is from an SV40 transfection, which effects cell cycle entry, no difference in proliferative potential between these two cell lines was identified. However, it should be noted that the cell line showing nuclear localization did show significantly lower light scattering, suggesting the nuclear ALDH1A1 may have dual functions regarding cell cycle entry and light scattering. Future studies evaluating the effects of ALDH1A1 and/or ALDH3A1 in cell cycle progression in keratocytes are needed.

Finally, there are several limitations to this study. First, the cell volume measurements were based on fluorescent imaging of calcein, and as such, the volumes measured are most likely representative and are not a true measure of the cell volume. Furthermore, significant quenching of calcein fluorescence is known to occur when high concentrations accumulate inside the cell and therefore differences in loading of different cell types may affect fluorescence intensity and the volume measurements. Since there is no reason to suspect that calcein would accumulate to greater concentration in either myofibroblasts or keratocytes, the relative differences measured in this study should be an accurate representation of the actual difference in cell volume. It should be noted, however, that the myofibroblasts appeared to contain ~50% more protein per cell volume than did the keratocytes. If the amount of protein per cell volume is constant to maintain osmotic equilibrium, then the differences in cell volume of keratocytes and myofibroblasts may be 50% greater.

A second limitation of this study is the measurement of single-cell light scattering. Reflectance confocal microscopy measures scattering 180° from the incident light and as such is not a true measure of light scattering. However, it should be noted that assessment of corneal haze as made by slit lamp examination for the most part uses backscattered light, and therefore the differences noted in the present study would be similar to that potentially observed in living corneas.

In conclusion, the data from this study indicate for the first time that myofibroblast differentiation leads to a dramatic increase in cell size and protein content that markedly dilutes the concentration of intracellular keratoocyte corneal crystallins. Based on these findings we propose that dilution of corneal crystallin reduces the protective and antioxidant effects of corneal crystallins leading to protein modification, aggregation, and increased light scattering. As such, the mechanism of haze in the cornea and cataract formation in the lens may have similar molecular mechanisms; that is, corneal crystallins serve a similar if not identical function to that of lens crystallins. Indeed, it is well recognized that many of the lens crystallins have been identified as corneal crystallins, suggesting a parallel evolutionary development. Further studies on corneal crystallins are needed to further test this hypothesis.

References

- Jester JV, Barry-Lane PA, Cavanagh HD, Petroll WM. Induction of alpha-smooth muscle actin expression and myofibroblast transformation in cultured corneal keratocytes. *Cornea*. 1996;15:505-516.
- Masur SK, Dewal HS, Dinh TT, Erenburg I, Petridou S. Myofibroblasts differentiate from fibroblasts when plated at low density. *Proc Natl Acad Sci U S A*. 1996;93:4219-4223.
- Berryhill BL, Kader R, Kane B, Birk DE, Feng J, Hassell JR. Partial restoration of the keratoocyte phenotype to bovine keratocytes made fibroblastic by serum. *Invest Ophthalmol Vis Sci*. 2002;43:3416-3421.
- Jester JV, Ho-Chang J. Modulation of cultured corneal keratoocyte phenotype by growth factors/cytokines control in vitro contractility and extracellular matrix contraction. *Exp Eye Res*. 2003;77:581-592.
- Jester JV, Huang J, Fisher S, et al. Myofibroblast differentiation of normal human keratocytes and hTERT, extended-life human corneal fibroblasts. *Invest Ophthalmol Vis Sci*. 2003;44:1850-1858.
- Skalli O, Ropraz P, Trzeciak A, Benzoni G, Gillesse D, Gabbiani G. A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol*. 1986;103:2787-2796.
- Petroll WM, Cavanagh HD, Barry P, Andrews P, Jester JV. Quantitative analysis of stress fiber orientation during corneal wound contraction. *J Cell Sci*. 1993;104:353-363.
- Grinnell F, Petroll WM. Cell motility and mechanics in three-dimensional collagen matrices. *Annu Rev Cell Dev Biol*. 2010;26:335-361.
- Jester JV, Petroll WM, Cavanagh HD. Corneal stromal wound healing in refractive surgery: the role of myofibroblasts. *Prog Retin Eye Res*. 1999;18:311-356.
- Møller-Pedersen T, Cavanagh HD, Petroll WM, Jester JV. Neutralizing antibody to TGFbeta modulates stromal fibrosis but not regression of photoablative effect following PRK. *Curr Eye Res*. 1998;17:736-747.
- Jester JV, Barry-Lane PA, Petroll WM, Olsen DR, Cavanagh HD. Inhibition of corneal fibrosis by topical application of blocking antibodies to TGF beta in the rabbit. *Cornea*. 1997;16:177-187.
- Cintron C, Szamier RB, Hassinger LC, Kublin CL. Scanning electron microscopy of rabbit corneal scars. *Invest Ophthalmol Vis Sci*. 1982;23:56-63.
- Cintron C, Hong BS, Kublin CL. Quantitative analysis of collagen from normal developing corneas and corneal scars. *Curr Eye Res*. 1981;1:1-8.
- Rawe IM, Meek KM, Leonard DW, Takahashi T, Cintron C. Structure of corneal scar tissue: an X-ray diffraction study. *Biophys J*. 1994;67:1743-1748.
- Lipshitz I, Loewenstein A, Varssano D, Lazar M. Late onset corneal haze after photorefractive keratectomy for moderate and high myopia. *Ophthalmology*. 1993;104:369-373, discussion 373-364.
- Shii D, Nakagawa S, Shinomiya K, et al. Cyclosporin A eye drops inhibit fibrosis and inflammatory cell infiltration in murine type I allergic conjunctivitis without affecting the early-phase reaction. *Curr Eye Res*. 2009;34:426-437.
- Nien CJ, Flynn KJ, Chang M, Brown D, Jester JV. Reducing peak corneal haze after photorefractive keratectomy in rabbits: prednisolone acetate 1.00% versus cyclosporine A 0.05%. *J Cataract Refract Surg*. 2011;37:937-944.
- Jester JV, Møller-Pedersen T, Huang J, et al. The cellular basis of corneal transparency: evidence for 'corneal crystallins'. *J Cell Sci*. 1999;112(Pt 5):613-622.
- Alexander RJ, Silverman B, Henley WL. Isolation and characterization of BCP 54, the major water soluble protein of bovine cornea. *Exp Eye Res*. 1981;32:205-216.
- Holt WS, Kinoshita JH. The soluble proteins of the bovine cornea. *Invest Ophthalmol Vis Sci*. 1973;12:114-126.
- Jester JV. Corneal crystallins and the development of cellular transparency. *Semin Cell Dev Biol*. 2008;19:82-93.
- Jester JV, Budge A, Fisher S, Huang J. Corneal keratocytes: phenotypic and species differences in abundant protein expression and in vitro light scattering. *Invest Ophthalmol Vis Sci*. 2005;46:2369-2378.
- Piatigorsky J. Enigma of the abundant water-soluble cytoplasmic proteins of the cornea: the "refraction" hypothesis. *Cornea*. 2001;20:853-858.
- Jester JV, Lee YG, Huang J, et al. Development of postnatal corneal transparency is associated with keratoocyte cell cycle exit and marked expression of ALDH1A1. *Invest Ophthalmol Vis Sci*. 2007;48:4061-4069.
- Sax CM, Kays WT, Salamon C, Chervenak MM, Xu YS, Piatigorsky J. Transketolase gene expression in the cornea is influenced by

- environmental factors and developmentally controlled events. *Cornea*. 2000;19:833–841.
26. Pei Y, Reins RY, McDermott AM. Aldehyde dehydrogenase (ALDH) 3A1 expression by the human keratocyte and its repair phenotypes. *Exp Eye Res*. 2006;83:1063–1073.
 27. Møller-Pedersen T. Keratocyte reflectivity and corneal haze. *Exp Eye Res*. 2004;78:553–560.
 28. Møller-Pedersen T, Li HF, Petroll WM, Cavanagh HD, Jester JV. Confocal microscopic characterization of wound repair after photorefractive keratectomy. *Invest Ophthalmol Vis Sci*. 1998;39:487–501.
 29. Brown DJ, Lin B, Holguin B. Expression of neuregulin 1, a member of the epidermal growth factor family, is expressed as multiple splice variants in the adult human cornea. *Invest Ophthalmol Vis Sci*. 2004;45:3021–3029.
 30. Spirin KS, Ljubimov AV, Castellon R, et al. Analysis of gene expression in human bullous keratopathy corneas containing limiting amounts of RNA. *Invest Ophthalmol Vis Sci*. 1999;40:3108–3115.
 31. Barry-Lane PA, Wilson SE, Cavanagh HD, Petroll WM, Jester JV. Characterization of SV40-transfected cell strains from rabbit keratocytes. *Cornea*. 1997;16:72–78.
 32. Lassen N, Pappa A, Black WJ, et al. Antioxidant function of corneal ALDH3A1 in cultured stromal fibroblasts. *Free Radic Biol Med*. 2006;41:1459–1469.
 33. Stagos D, Chen Y, Cantore M, Jester JV, Vasiliou V. Corneal aldehyde dehydrogenases: multiple functions and novel nuclear localization. *Brain Res Bull*. 2010;81:211–218.
 34. Beales MP, Funderburgh JL, Jester JV, Hassell JR. Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: maintenance of the keratocyte phenotype in culture. *Invest Ophthalmol Vis Sci*. 1999;40:1658–1663.
 35. Karring H, Thøgersen IB, Klintworth GK, Enghild JJ, Møller-Pedersen T. Proteomic analysis of the soluble fraction from human corneal fibroblasts with reference to ocular transparency. *Mol Cell Proteomics*. 2004;3:660–674.
 36. Stramer BM, Cook JR, Fini ME, Taylor A, Obin M. Induction of the ubiquitin-proteasome pathway during the keratocyte transition to the repair fibroblast phenotype. *Invest Ophthalmol Vis Sci*. 2001;42:1698–1706.
 37. Stramer BM, Fini ME. Uncoupling keratocyte loss of corneal crystallin from markers of fibrotic repair. *Invest Ophthalmol Vis Sci*. 2004;45:4010–4015.
 38. Lassen N, Black WJ, Estey T, Vasiliou V. The role of corneal crystallins in the cellular defense mechanisms against oxidative stress. *Semin Cell Dev Biol*. 2008;19:100–112.
 39. Manzer R, Pappa A, Estey T, Sladek N, Carpenter JF, Vasiliou V. Ultraviolet radiation decreases expression and induces aggregation of corneal ALDH3A1. *Chem Biol Interact*. 2003;143–144:45–53.
 40. Manzer R, Qamar L, Estey T, Pappa A, Petersen DR, Vasiliou V. Molecular cloning and baculovirus expression of the rabbit corneal aldehyde dehydrogenase (ALDH1A1) cDNA. *DNA Cell Biol*. 2003;22:329–338.
 41. Pappa A, Brown D, Koutalos Y, DeGregori J, White C, Vasiliou V. Human aldehyde dehydrogenase 3A1 inhibits proliferation and promotes survival of human corneal epithelial cells. *J Biol Chem*. 2005;280:27998–28006.
 42. Estey T, Cantore M, Weston PA, Carpenter JF, Petrash JM, Vasiliou V. Mechanisms involved in the protection of UV-induced protein inactivation by the corneal crystallin ALDH3A1. *J Biol Chem*. 2007;282:4382–4392.
 43. Bloemendal H, de Jong W, Jaenicke R, Lubsen NH, Slingsby C, Tardieu A. Ageing and vision: structure, stability and function of lens crystallins. *Prog Biophys Mol Biol*. 2004;86:407–485.
 44. Lassen N, Bateman JB, Estey T, et al. Multiple and additive functions of ALDH3A1 and ALDH1A1: cataract phenotype and ocular oxidative damage in *aldh3a1*(–/–)/*aldh1a1*(–/–) knockout mice. *J Biol Chem*. 2007;282:25668–25676.
 45. Chen Y, Koppaka V, Thompson DC, Vasiliou V. Focus on Molecules: ALDH1A1: From lens and corneal crystallin to stem cell marker. *Exp Eye Res*. Published online April 23, 2011.