Lens cytoskeleton and transparency: a model

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Abstract

The function of the cytoskeleton in lens was first considered when cytoplasmic microtubules were observed in elongating fibre cells of the chick lens nearly 40 years ago.¹ Since that time, tubulin, actin, vimentin and intermediate filaments have been identified and found to function in mitosis, motility and cellular morphology during lens cell differentiation.^{2–10} A role for the cytoskeleton in accommodation has been proposed^{3,8,9} and modification of the cytoskeletal proteins has been observed in several cataract models.^{4,11–21} Recently, a progressive increase in protein aggregation and lens opacification was found to correspond with the loss of cytoskeletal protein in the selenite model for cataract.²² In the present report a model is proposed for the role of tubulin, actin, vimentin, spectrin and the lens-specific filaments, filensin and CP49, in the establishment and maintenance of transparent lens cell structure.

Key words Cataract, Cytoskeleton, Lens, Opacity, Transparency

The primary function of the normal transparent lens is to transmit and focus visible light ($400\text{ nm} < \lambda_{\text{vis}} < 700\text{ nm}$). Focus is associated with the symmetrical shape and index of refraction of the lens. Synchronised proliferation and differentiation of layers of lens cells may be responsible for the beautiful symmetry of the histological structure of the biological lens.^{23–27} This symmetry is disturbed when abnormal proliferation, migration and elongation of lens cells alter development and synchronous differentiation, ultimately causing abnormal focus. While coordinated proliferation and differentiation of lens epithelial cells involve the cytoskeleton,^{6,8,12} the presence of organelles in lens fibres impairs transmission of visible light. Normal differentiation eliminates nuclei, mitochondria, Golgi bodies, endoplasmic reticulum and intracellular structures that are large enough to scatter light and contribute to opacity.^{28–30} The synchronised and coordinated differentiation of lens fibres is accompanied by an increase in lens protein concentrations to levels necessary for a high refractive index. The rapid expression and

concentration of crystallin proteins occurs at the lens periphery in differentiating epithelial cells,^{29,31,32} where important molecular and cellular mechanisms regulate the normal folding of crystallins and their interactions. Elevated protein concentrations favour protein aggregation, but in normal lens cells the energies of interaction between cytoplasmic proteins favour a single homogeneous transparent phase.^{33–35} When the energies of interaction favour organisation of cellular proteins into separate cytoplasmic domains of condensed protein aggregates, cataracts appear. In cataract, the cellular opacity is a function of the dimensions and the number of the aggregates. If it is assumed that weak non-covalent interactions are responsible for transparent organisation of cytoplasmic proteins,³⁶ it is understandable that even the simplest modification in the proteins or their environment can influence the transparency or opacity of differentiating lens fibres. It is remarkable that the interactions between lens proteins and their environment favour short-range, glass-like order and cellular transparency even at the extremely high protein concentrations found in lens cells.^{33,37–41} We suggest a role for the cytoskeleton in the development and maintenance of lens transparency.

In mammals, the ability to organise cellular proteins into a transparent, glass-like optical element is unique to the developing lens. Lens cells contain the cytoskeletal elements found in most epithelial cells as well as intermediate filaments known to be lens-specific.^{42–45} Lens cells are transparent because of short-range order in the organisation of cytoplasmic proteins, which are largely crystallins.^{37,40} It should be noted that lens proteins are known as crystallins although the organisation of the crystallin proteins in lens cytoplasm is non-crystalline short-range order. The establishment of short-range order in lens cytoplasm may involve interactions between lens crystallins and the prominent cytoskeletal proteins to provide an environment favourable for transparent cell structure.²² The cytoskeletal and filamentous structures are large enough to contribute to light scattering,⁴⁶ and elimination of cytoskeletal and filamentous structures may be necessary once short-range order is established in differentiating fibres. Because

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Support was provided by
grant #04542 from the
National Eye Institute of NIH

lens cell differentiation includes elimination of organelles large enough to scatter light, similar mechanisms may act to eliminate filaments and cytoskeletal elements during development.^{47–51} In rat, the calcium-sensitive protease, calpain, modifies crystallins during normal lens development. Previous studies in lenses of normal rats found a slow continuous loss of the N-terminal extensions of β -crystallin that accelerated during opacification in selenite cataract.^{49,52} We observed a similar but more rapid decrease in cytoskeletal proteins and lens-specific filaments during development of the normal transparent lenses. Accelerated and abnormal degradation of these same lens proteins was observed during opacification in the selenite cataract model. In lenses of animals administered the anti-cataract agent, pantethine, the partial degradation of β -crystallins and the loss of the cytoskeleton and lens-specific filaments was inhibited and transparency was maintained.

Methods

Ten-day-old Sprague-Dawley rat pups and their mothers were obtained from B&K Universal (Kent, WA) and prepared for experiments as described previously.²² All investigations conformed to the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals in Research and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. D-Pantethine syrup and selenite were obtained from Sigma Chemical (St Louis, MO). The pantethine syrup was so viscous that it was transferred using a microspatula. One gram of pantethine was brought to a volume of 2 ml in a volumetric flask with distilled water. Pantethine was completely dissolved with gentle mixing and was administered immediately to rat pups. The subcutaneous dose of pantethine was 820 mg kg⁻¹ rat body weight (1.5 mmol kg⁻¹). The pH was 6.8 for a 410 mg ml⁻¹ solution. To initiate cataract, the rat pups were injected subcutaneously in the shoulder on postnatal day 13 or 14 with a solution of 1.8 mg ml⁻¹ selenite dissolved in 0.9% sodium chloride (pH 9) to give a dose of 3.28 mg kg⁻¹ (19 μ mol kg⁻¹). To inhibit cataract formation, pantethine was injected into the opposite shoulder approximately 30 min before the selenite injection. Control animals received an injection of buffer without selenite or pantethine.

The eyes of the injected rats were examined using a photo slit lamp microscope (Nikon FS-2; Nikon, Melville, NY) at selected time points and graded using the classification described previously.⁵³ Prior to the examination, the pupils were dilated with a drop of 1:1 mixture of 1% tropicamide (Schein Pharmaceuticals, Florham Park, NJ) and 10% phenylephrine hydrochloride (Akorn, Abita Springs, LA).

Rats were killed on days 0, 1, 2, 3 and 4 after injection and their lenses were dissected into cortex and nucleus as described previously.²² To separate the lens samples into soluble and insoluble protein, a 150 μ l solution of 20 mM sodium phosphate, 1.0 mM EGTA (pH 7.0) was added to each sample. Each lens was homogenised in a

microhomogeniser and the homogenates were centrifuged for 15 min at 12 000 g at 4 °C. The supernatants were removed and placed in new tubes and the insoluble pellets were then resuspended in 150 μ l of buffer. This process was repeated twice. The remaining pellets were then dissolved in 60 μ l of 8 M urea solution. The BCA (bicinchoninic acid) reagent (Pierce, Rockford, IL) was used for assaying protein concentration using bovine serum albumin as a standard. Proteins were analysed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% Tris-glycine precast gradient mini-gels (Novex, San Diego, CA) as described previously²² (Fig. 1).

To identify high-molecular-weight (HMW) proteins, Western immunoblot analysis was performed using a Blot module with nitrocellulose or PVDF membranes (Novex) as described previously.²² The following were used as primary antibodies: monoclonal anti-actin (ICN,

Fig. 1. Method for analysis of lens proteins. Intact lenses were dissected into nuclear and cortical fractions that were diluted with 20 mM phosphate buffer and homogenised. The homogenates were centrifuged to produce a soluble supernatant and an insoluble pellet that was solubilised using 8 M urea. The protein constituents in the four fractions were separated using SDS-PAGE that permitted quantification of changes in the components. Electrophoresis and Coomassie blue staining of the lens extracts demonstrated that the major protein constituents are crystallins of 17 to 38 kDa. At higher molecular weights prominent bands were identified using Western blotting.

Costa Mesa, CA); monoclonal anti- α -tubulin; monoclonal anti-vimentin; polyclonal anti-spectrin (Sigma). Antibodies to the lens-specific filaments filensin and CP49 (phakinin) were generously supplied by Dr Roy Quinlan. For secondary antibody and immunodetection, alkaline phosphatase conjugated anti-mouse or anti-rabbit IgG antibody and BCIP/NBT (5-bromo-4-chlor-3-indolyl phosphate/nitro-blue tetrazolium chloride) Bio-Rad (Hercules, CA) were used.

To analyse small changes in the protein bands separated using SDS-PAGE, densitometry was conducted on the stained and dried gels. The dried gels were digitised and stored as 8 bit/pixel grey-scale image files. The program NIH IMAGE 1.58 was used for the analysis of each lane of the digitised images of the gels. A density profile of each lane of the gel was used to calculate the peak area for selected protein bands of 42, 49, 90 and 235 kDa in the lens nucleus and for 42, 49, 55/57, 60, 90 and 235 kDa in the lens cortex. The area of each selected peak was normalised to the total area of all peaks in a lane to obtain the proportion of protein in the selected band on each day. The percentages listed in Table 1 were calculated as follows: (the proportion on day 'd')/(the proportion of the same protein on day 1) $\times$ 100%, where 'd' is day 1, 2, 3 or 4.

Results

In the selenite model, there was a rapid progression of protein aggregation to full nuclear opacity by 7 days after injection of selenite^{22,53} (Fig. 2). Pantethine inhibited protein aggregation and opacification when administered early in the formation of the cataract. The major proteins in the samples from young rat lenses were identified using SDS-PAGE gels stained with Coomassie blue. Crystallins appeared as low-molecular-weight proteins < 40 kDa and non-crystallin proteins appeared at high molecular weights > 40 kDa (Fig. 3). Using antibodies to the major cytoskeletal proteins in the lens, the HMW proteins were identified as spectrin, vimentin, tubulin, actin and the lens-specific filaments, filensin and CP49, in Western immunoblots. Only the 60 kDa band remained unidentified. More than a single immunoreactive band appeared in the spectrin, vimentin and filensin lanes, confirming the processing or degradation of the cytoskeleton that was reported during normal lens cell differentiation⁴⁵ (Fig. 3). Once the identity of the proteins was established, quantification was carried out using densitometry.

Table 1 lists the quantitative changes in the prominent HMW protein bands observed in SDS-PAGE gels of homogenates from the lens nucleus and cortex for days 1-4 after injection of selenite to induce the cataract. It should be noted that formation of a dense nuclear cataract was just beginning at day 4 and had not reached maturity when observed in a slit lamp.^{22,53,54} In the lenses from normal animals the progressive decrease in the HMW proteins suggested processing and degradation of the cytostructural proteins during normal development of lens transparency. This loss was most

Fig. 2. Transparent and opaque lenses from the rat. Anterior photographs and slit lamp views of the eyes of control animals (a, b), selenite-injected animals (c, d) and pantethine + selenite-injected animals (e, f) demonstrate the obvious dense nuclear opacity that is characteristic of the selenite cataract. Following selenite injection there is a progressive increase in the aggregated and insoluble protein and loss of transparency due to light scattering (c, d). Pantethine inhibited protein aggregation and opacification, which resulted in nearly complete protection against cataract formation in animals injected with selenite (e, f). Photographs were taken 14 days after injection of the animals.

evident in the lens nucleus. In the animals receiving selenite, the loss of the HMW proteins and β B₁-crystallin was dramatic. By day 3, spectrin, filensin and intact β B₁-crystallin levels had fallen to undetectable levels and other proteins had decreased 50% or more. In the lenses of the selenite + pantethine group, in which lenses remained transparent, the levels of the HMW bands were similar to the controls and much greater than in the lenses from the animals that were administered selenite only. At some time points, increases in the proteins were measured. For example, on day 2, spectrin and actin in normal lens cortex increased to 110% and 116%, respectively, relative to day 1 and β B₁-crystallin increased in the normal nucleus to 114% on day 2. While it is possible that differences in preparation account for part of the increase, protein concentrations are known to oscillate in developing lenses.^{23,24}

Discussion

Lens-specific filament and cytoskeletal proteins are prominent components of the insoluble fraction of both cortical and nuclear cells.^{2,12,22,43,55} As a result of the preferential expression of crystallins during differentiation, the relative proportion of cytoskeletal

Fig. 3. Western immunoblots using antibodies to structural proteins in the insoluble fraction of the lens cortex (upper figure) and nucleus (lower figure). The left-hand lane contains the standard proteins with the approximate molecular weights listed. Lane 2 is a Coomassie blue stained SDS-PAGE of the insoluble cortex (IC) or insoluble nucleus (IN) from the lens of a normal animal. The six lanes to the right show the results of immunoblotting with anti-actin (A, 42 kDa), anti-tubulin (T, 55 kDa), anti-vimentin (V, 57 kDa), anti-spectrin (S, 235 kDa), anti-filensin (F, 90 kDa) and anti-CP49 or phakinin (P, 49 kDa).

proteins is expected to decline slightly in normal lens cells. The observed decrease in the cytostructural proteins in the normal lens without selenite is greater than expected when considering dilution and suggests there may be progressive degradation, possibly due to proteolytic processing, during normal differentiation of transparent lens cells. One interpretation of these results is related to the function of cytoskeletal proteins in the establishment of cellular transparency. Interactions between cytostructural proteins and crystallins may be required for the initial organisation of lens proteins into the transparent homogeneous structure necessary for maintenance of transparency. Once the transparent structure of a lens cell is established, the cytoskeletal elements that are large enough to scatter light may be degraded to eliminate their light-scattering potential.

Prior to the appearance of opacity in the selenite-treated animals, protein modifications can be observed in the large HMW bands and in β -crystallins using SDS-PAGE and two-dimensional electrophoresis analysis of extracts of lens proteins.^{49,56} In the absence of a cytoskeletal scaffold, attractive interactions between lens proteins may dominate and accelerate the organisation of proteins into large light-scattering aggregates. The anti-cataract effect of pantethine inhibited both protein degradation and opacification. It appears that complete protection of the cytoskeleton may be unnecessary for inhibition of protein aggregation and that the effect of pantethine may have been stronger if multiple doses had been administered instead of a single dose. A model for the role of the cytoskeleton in lens transparency is presented in Fig. 4.

Table 1. Results of quantitative analysis of lens proteins

Molecular weight	Group	Nucleus				Cortex			
		Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
<i>(a) > 40 kDa</i>									
235 kDa (spectrin)	Normal	100%	77%	69%	46%	100%	110%	108%	104%
	Se	100%	29%	0	0	100%	56%	30%	46%
	Pa + Se	100%	113%	38%	38%	100%	81%	94%	82%
90 kDa (filensin)	Normal	100%	92%	76%	40%	100%	95%	100%	105%
	Se	100%	67%	0	0	100%	74%	91%	70%
	Pa + Se	100%	88%	38%	42%	100%	104%	126%	96%
60 kDa	Normal	None	None	None	None	100%	85%	76%	73%
	Se					100%	72%	25%	19%
	Pa + Se					100%	98%	102%	73%
55/57 kDa (vimentin and tubulin)	Normal	None	None	None	None	100%	105%	93%	103%
	Se					100%	80%	61%	43%
	Pa + Se					100%	89%	102%	100%
49 kDa (CP49, phakinin)	Normal	100%	91%	82%	49%	100%	87%	87%	83%
	Se	100%	85%	49%	44%	100%	83%	102%	85%
	Pa + Se	100%	115%	62%	85%	100%	87%	94%	84%
42 kDa (actin)	Normal	100%	110%	90%	80%	100%	116%	118%	109%
	Se	100%	128%	33%	29%	100%	75%	79%	80%
	Pa + Se	100%	140%	110%	150%	100%	85%	100%	100%
<i>(b) < 40 kDa</i>									
30 kDa (βB1) soluble protein	Normal	100%	114%	86%	91%	100%	98%	100%	95%
	Se	100%	108%	0	0	100%	104%	88%	82%
	Pa + Se	100%	94%	78%	94%	100%	110%	113%	105%
30 kDa (βB1) insoluble protein	Normal	100%	97%	84%	62%	100%	95%	89%	80%
	Se	100%	93%	0	0	100%	98%	78%	79%
	Pa + Se	100%	95%	64%	59%	100%	100%	100%	106%

Selected proteins were identified on Western immunoblots and quantified in SDS-PAGE gels of homogenates from lenses removed from control (Normal), selenite injected (Se) and pantethine + selenite injected (Pa + Se) rats. The molecular weights and identity, based on immunoreactivity, are listed in column 1 and the treatment groups are listed in column 2. Columns 3 to 6 list the results for proteins in the insoluble fraction from the nucleus, and columns 7 to 10 list the results for proteins in the insoluble fraction from the cortex. The upper table lists the results for the proteins of molecular weight > 40 kDa and the lower table for proteins < 40 kDa that were of interest. The days after initiation of the experiment (day of injection of selenite, buffer or pantethine + selenite) when the lenses were removed are listed at the top of each column. The results are presented as percentages of the protein content measured on day 1. 'None' indicates that the proteins were absent in the lens samples.

Rat models for cataract are commonly used in studies of lens development and/or lens opacification. An advantage of the selenite model is that the young age of the animals permits comparison of mechanisms that are important for normal development of transparency with mechanisms leading to protein aggregation and opacification. The rapid and reproducible occurrence of the opacification means that repeated experiments can be conducted in a relatively brief time period. While the cataract appears in a few days, many of the molecular events in the selenite model correspond with those in the ageing human, including post-translational modification and aggregation of lens proteins, altered calcium and glutathione levels, abnormal ionic composition and proteolysis.^{54,57} The importance of proteolysis as a mechanism of protein modification in lens cells during cataract formation has been investigated thoroughly using the selenite model, and modification of the cytoskeletal proteins accompanies alterations of the crystallins that occur early in the formation of the cataract and well before visible opacity is observed.^{22,49,56} In the normal lens, cytoskeletal proteins and selected crystallins appear to be substrates for the same

proteolytic enzymes that result in post-translational modification and altered interactions between proteins.^{14,17,47,52,58–60} It is well established that abnormal interactions between cytoplasmic constituents lead to protein aggregation and opacification in numerous animal models for cataract including the selenite model in the rat.^{61,62} Taken together, the findings suggest that modification of cytoskeletal elements as well as modification of crystallins may contribute to the interactions responsible for the early stages of protein aggregation and opacification. However, the functional importance of proteolytic modification during normal development of lens transparency has received little attention. Proteolytic modifications that stabilise interactions required for transparency would be advantageous, especially in the absence of structural support provided by the cytoskeleton.

Previous studies of interactions in normal lenses and during cataract formation used the phase separation temperature, T_c , as a direct measure of the energies of interaction between lens cell constituents.^{34,35,63} T_c is defined by the expression:

Fig. 4. A model for the function of the cytoskeleton in the establishment and maintenance of lens cell transparency. In a transparent lens cell, the distribution of cytoplasmic proteins is uniform and homogeneous. The symmetrical and transparent structure of the lens is the result of a coordinated process of epithelial cell differentiation that includes elimination of large light-scattering organelles and the concentration of lens crystallins to very high levels. High protein concentrations favour protein aggregation in non-lens cells. Differentiating lens cells are rich in cytoskeletal proteins that can interact with crystallins as they are expressed during development of lens transparency (top panel). The cytoskeletal elements may participate in the establishment of transparent structure by providing a scaffold for organisation of crystallins into a homogeneous transparent cell structure. Progressive degradation of the filaments and cytoskeletal elements that are large enough to scatter light may occur after transparency has been established. In some lenses, smaller elements of the cytoskeleton may persist to maintain the transparent lens cell structure. In cataract involving the cytoskeleton, abnormal modification of the cytoskeletal proteins or abnormal acceleration of the degradation process may alter the function of the cytoskeleton in transparency. In the absence of a functional cytoskeleton, the attractive forces between lens proteins can cause separation of the cytoplasm into distinct protein-poor and protein-rich phases that are large with respect to the wavelength of visible light (middle panel). The administration of pantethine, an anti-cataract agent that acts to inhibit interactions leading to protein aggregation, protected against the loss of cytoskeletal proteins and opacification in the selenite model (bottom panel). In many cases pantethine administration resulted in partial protection of the cytoskeleton indicating inhibition of protein aggregation may not require complete protection of all elements of the entire cytoskeleton. Taken together, these findings support the hypothesis that interactions between the cytoskeleton and lens crystallins are involved in the establishment and maintenance of lens cell transparency.

$$kT_c = A [E_{pw} - (E_{ww} + E_{pp})/2]$$

where k is the Boltzmann constant, T_c phase separation temperature, which is a function of E_{pw} , the protein-water energy of interaction, E_{ww} , the water-energy of

interaction, E_{pp} , the protein-protein energy of interaction, and A is a thermodynamic constant that is defined by the form of the phase diagram of lens cytoplasmic proteins. While it is an oversimplification, the expression for T_c includes a term for the single homogeneous phase and a term for separated condensed and dilute protein phases. Modifications of composition that favour protein condensation and aggregation increase the latter term that is measured as an abnormal increase in T_c which is predictive of protein aggregation and lens opacification. For example, as the crystallins increase in concentration across the radius of the lens, there is a corresponding increase in T_c because high protein concentrations favour protein aggregation.^{33,38,64} In this report, we emphasise that an interaction between the cytoskeleton and the crystallins may be at least as important as the interactions between crystallins in the development and maintenance of lens cell transparency.

In several animal models for cataract formation involving post-translational modification and protein aggregation, T_c increased well before the appearance of the cataract *in vitro*.⁶¹⁻⁶⁴ In differentiated fibre cells, the total concentration of proteins is 4 to 8 times the protein concentrations found in non-lens cells. More than 90% of the proteins in lens are crystallins.⁶⁵ Given the strong effect of protein concentration on T_c ,³⁴ non-crystallin constituents of differentiating lens cells may participate in the organisation of transparent cytoplasm to maintain low T_c . Of the non-crystallin proteins, more than half are HMW structural proteins including actin, tubulin, vimentin, spectrin and the lens-specific filaments, filensin and phakinin.^{42,44} If the concentration of crystallin proteins were reduced to the concentration of cytoplasmic proteins found in most epithelial cells, the levels of filaments and cytoskeletal proteins would be exceptionally high, possibly higher than the concentrations found in cells containing cilia and flagella. Lens cytoskeletal proteins are involved in proliferation, migration and elongation of fibre cells during normal development. In recent studies, interactions between cytoskeleton and crystallin proteins have been observed,⁶⁶⁻⁷¹ but precisely how the cytoarchitecture influences interactions between crystallins during differentiation of transparent lens cells remains to be defined. The present report summarises changes in cytoskeletal proteins observed during normal development of the transparent lens and in the selenite model for cataract formation, where a dramatic loss of cytoskeletal protein is among the earliest modifications observed using SDS-PAGE gel electrophoresis. The relevance of cytoskeletal modification in human cataract and in establishment and maintenance of transparency in the human lens needs to be determined.

We are grateful for the generous assistance of P. Muchowski, Dr T. Hiraoka, Dr R. Quinlan and Ms C. Ganders. Supported by E404542 from the NEI.

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