Proteomic Analysis of Human Age-related Nuclear Cataracts and Normal Lens Nuclei

Sheng Su,1 Ping Liu,1 Hong Zhang,1 Zhijian Li,1 Zhen Song,1 Lu Zhang,1 and Sbuto Chen2

PURPOSE. To identify proteomic differences between age-related nuclear cataracts (ARNCs) and normal lens nuclei.

METHODS. Total solubilized proteins from ARNC lens nuclei with different grades were compared with normal controls by 2-D differential in-gel electrophoresis (2-D DIGE). Proteins with different abundances were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses determined the compositions of high molecular weight (HMW; >200 kDa) aggregates found in ARNC lens nuclei. Western blot analysis was used to verify the changes in αA-crystallin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels.

RESULTS. The 2-D differential in-gel electrophoresis results showed that nine proteins were significantly less abundant in lens nuclei from ARNC patients than in control lens nuclei. Six proteins (αA-, βA3-, βA4-, βB1-, and γD-crystallin and putative uncharacterized protein DKFZp434A0627) from the CHYGS family tended to decrease as the cataract grade increased, while the other three proteins (αB-crystallin, GAPDH, and retinal dehydrogenase 1) did not show such a tendency. SDS-PAGE showed decreased protein levels at ~20 kDa in ARNC lenses but significantly increased levels at HMW (>200 kDa). Liquid chromatography tandem mass spectrometry analysis showed that the HMW aggregates derived largely from crystallins also contained filensin, phakinin, and carbonyl reductase 1. Of all the components, αA-crystallin accounted for the highest fraction. αA-, αB-, and γD-crystallin and DKFZp434A0627 were more prone to aggregate than other crystallins.

CONCLUSIONS. The results show that crystallins, especially αA-crystallin, aggregate irreversibly during ARNC development. Some enzymes (GAPDH, retinal dehydrogenase 1, and carbonyl reductase 1) may be involved in and/or accelerate this process. (Invest Ophthalmol Vis Sci. 2011;52:4182–4191) DOI:10.1167/iovs.10-7094

The lens is a cellular organ, and its transparency and stability are achieved by tightly packing crystalline proteins into a glass-like microarchitecture.1 Any changes affecting the structure or amount of specific crystallins can lead to a cataract. The incidence of different cataract type increases with age: nuclear cataracts, which account for approximately 60% of age-related cataracts; cortical cataracts, which account for approximately 30%; and posterior subcapsular cataracts, which account for the remaining 10%.2 Age-related nuclear cataracts (ARNCs) are characterized by opacification of the lens nucleus, which is the center of the lens.

The crystallins, α-, β-, and γ-crystallin, account for approximately 90% of the total protein in lens fiber cells. The human crystallins, which include the αA, αB, βB1, βB2, βB3, βA3/A1, βA2, βA4, γA, γB, γC, and γD forms, cannot be replaced and thus must last the entire lifetime of an individual. Over time, these lens proteins can undergo a variety of modifications, such as oxidation, deamidation, or cleavage, which result in incorrect protein-protein interactions and/or protein aggregation; these interactions and aggregations eventually cause the opacification of the lens. The α-crystallin molecule has a dual role as the main protein chaperone system in mature fiber cells and as a structural protein along with β- and γ-crystallin.3–7 Studies using recombinant proteins have confirmed that αA- and αB-crystallin interact with many partially denatured proteins.8–13

Human crystallin proteins start off largely water soluble; with maturation, they become water insoluble but urea soluble or even urea insoluble. α-crystallin gradually changes from being a water-soluble assembly of around 800 kDa formed from 20 kDa subunits to a state where it is no longer water soluble but is urea soluble. This transition may populate a higher molecular weight (soluble) assembly. Multicrystallin complexes, held together mostly via noncovalent bonding, are present in the water-soluble high molecular weight (HMW) protein fractions of cataractous and aging normal human lenses.14 Covalent multimers of crystallins are present in the water-insoluble proteins of aging human lenses.14 However, to date, little information has been reported about HMW protein aggregates and their compositions in ARNC lenses that have been solubilized with powerful deaggregating reagents. Because of the reversibility from aggregates to separated proteins in ARNC lenses, these HMW aggregates may be critical to cataract development.

Two-dimensional gel electrophoresis (2-DE) studies have demonstrated that the crystallins of human clear lenses undergo age-related changes.16–17 A recent study compared the water-insoluble proteins from the healthy lens of a 68-year-old donor with those from the cataractous lens of a 61-year-old patient.18 Ten percent of the cortex and 90% of the nucleus were used for research in that study. Two-dimensional gel electrophoresis proteome maps of total proteins and the differences between human normal and ARNC lens nuclei have not been elucidated. Furthermore, few studies have shown common changes or tendencies in cataractous lenses with different grades.

Thus, in this study, reproducible human lens nucleus 2-DE maps were created. Proteomic contents of normal and ARNC...
lens nuclei were compared and analyzed using 2-D differential in-gel electrophoresis (2-D DIGE) with respect to cataract grade. Proteins with different abundances were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Further, using liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses, we identified the components of an ARNC pooled sample, a normal pooled control, and the HMW aggregates found in ARNC lens nuclei, and the relative abundance of each component was estimated. These data may be helpful in studying the biochemical mechanisms of human ARNC.

**Materials and Methods**

**Tissue Collection**

All tissues used for this study were collected with prior approval from the Regional Committee for Scientific Medical Ethics in Heilongjiang Province in China, and the research adhered to the tenets of the Declaration of Helsinki. Seven ARNC lenses (age, 54 to 59 years old) and seven age-matched normal lenses (age, 53 to 59 years old) were obtained from the Eye Bank of the Eye Hospital, Harbin Medical University. Normal lenses were retrieved within 24 hours postmortem, and the analysis of these lenses did not reveal any opacity, thereby confirming their normal status. The presence of nuclear cataracts in the lenses removed from the ARNC patients was also confirmed. The nuclei was separated from the cortex at 4°C using needles and then stored at −80°C until use. The ARNC lens nuclei were graded based on color, as described by Pirie.19 Two ARNC nuclei were grade II with a visible nucleus (referred to as F and G), four nuclei were grade III with a hazel-brown nucleus (referred to as A, B, C, and D), and one nucleus was grade IV with a deep brown nucleus (referred to as E). In addition, the severity of the cataract increased as the grade increased.

**Sample Preparation**

Each frozen lens nucleus was ground in liquid nitrogen and then dissolved in 1 mL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris [pH 8.5], 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, and 1 µg/mL leupeptin). Thiourea, introduced by Ribiloud,20 is better suited than urea for breaking hydrophobic interactions and improving the solubilization of hydrophobic proteins. CHAPS (a zwitterionic detergent) was used to prevent hydrophobic interactions between the hydrophobic protein domains and avoid loss of proteins due to aggregation and precipitation.21 The combination of urea and thiourea chaotropes solubilizes at least several integral membrane proteins.22 The mixture was sonicated on ice and centrifuged at 4°C for 40 minutes. The supernatant was collected, and the protein concentration was determined according to the Bradford method. The protein concentration of each sample (A–G) and of the protein standard was determined according to the Bradford method. The protein concentration of normal and ARNC samples were 11.4 to 13.2 mg/mL and 12.1 to 14.5 mg/mL, respectively. There was no significant difference between solubilizable protein amounts obtained from clear versus cataractous lenses. Equal amounts of protein from each of the normal lens nuclei were pooled together, and this homogeneous mixture was referred to as N. Similarly, equal amounts of protein from each of the ARNC lens nuclei were pooled together and referred to as M. The protein concentration of each sample (A–G) and of the protein mixtures (N and M) was adjusted to 5 mg/mL with lysis buffer, and the pH was adjusted to 8.5 with 50 mM NaOH.

**Two-Dimensional Differential In-gel Electrophoresis (2-D DIGE) and Image Analysis**

A pooled sample consisting of equal aliquots of each sample (N and M) was used as an internal standard for quantitative analysis. The inclusion of an internal standard in 2-D gels eliminates errors resulting from electrophoresis artifacts and enables accurate quantitative analysis.23 The internal standard was labeled with Cy2 dyes, and the protein samples (N, M, and A–G) were labeled with Cy3 or Cy5. An additional reverse-labeling experiment was conducted for eight of the 2-D DIGE analyses. The 2-D DIGE experimental design is presented in Table 1. The labeling and 2-DE procedures were previously described.24 The pH range of the 24-cm immobilized nonlinear pH gradient (IPG) strips used in this study was 3 to 10. After 2-DE, the gels were scanned (Typhoon 9410 scanner; GE Health Care, Piscataway, NJ), and the Cy2, Cy3, and Cy5 fluorescence intensities of each gel were then individually imaged and analyzed (DeCyder 6.5 software; GE Health Care), according to the manufacturer’s instructions.

**Protein Identification by MALDI-TOF Mass Spectrometry**

Spot-picking and digestion were performed using preparative gels. The IPG strips (pH 3–10) were loaded with 1000 µg protein, and the gels were stained with Coomassie brilliant blue. After the preparative gels were scanned, protein spots of interest were excised from the gels and digested with trypsin. The tryptic peptides were extracted and dried completely by centrifugal lyophilization.

MALDI-TOF mass spectrometry and tandem TOF/TOF mass spectrometry were performed (4800 Plus MALDI TOF/TOF Instrument; Applied Biosystems, Foster City, CA), and identical procedures were used as previously described.24 Combined mass and mass/mass spectra were used to interrogate human sequences in the International Protein Index database (IPI_Human V3.53) using the Mascot database search algorithms. Confident protein identification had a statistically significant (P < 0.05) protein score and the best ion score.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Image Analysis**

Each protein sample (25 µg protein per lane) was resolved by non-continuous SDS-PAGE, using a 4% stacking gel and a 12% resolving gel. The ratio of acrylamide to bis-acrylamide was 30:0.8. After electrophoresis, the gels were stained with Coomassie brilliant blue and then scanned and image analysis was performed (ImageQuant TL software; Amersham Biosciences, Piscataway, NJ).

**Liquid Chromatography (LC)-MS/MS Analysis**

Based on image analysis, two slices (see Fig. 4A) were manually cut from the gel for an LC/MS/MS study. Protein samples (M and N) were also included in the LC/MS/MS analysis as the control of slice 2. The proteins in M, N, and the two slices were reduced in 10 mM dithiothreitol (DTT) at 56°C for 1 hour, alkylated in the dark with 55 mM iodoacetamide at room temperature for 1 hour, and then digested with trypsin. Tryptic peptides were dried completely by centrifugal lyophilization.

LC/MS/MS experiments were performed on a mass spectrometer (Finnigan LTQ Linear Ion Trap Mass Spectrometer; Thermo, San Jose, CA), equipped with a nanospray source and high-performance liquid chromatography (Eksigent NanoLC-2D; Eksigent Technologies, Liver-

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<th>Cy3</th>
<th>Cy5</th>
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<td>Internal Std</td>
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<td>M</td>
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<td>G</td>
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<td>Internal Std</td>
<td>B</td>
<td>A</td>
</tr>
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<td>B</td>
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<td>9</td>
<td>Internal Std</td>
<td>G</td>
<td>F</td>
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</table>

Internal Std denotes internal standard, which was a homogeneous mixture of equal amounts of N and M.
more, CA). The peptide mixture was separated on a fused silica microcapillary column with an internal diameter of 75 μm and an in-house prepared needle tip with an internal diameter of approximately 15 μm. Columns were packed to a length of 10 cm with a C18 reversed-phase resin (particle size 5 μm, pore size 300 Å). Separation was achieved using a mobile phase from 1.95% acetonitrile (ACN), 97.95% H2O, 0.1% formic acid (FA; phase A) and 79.95% ACN, 19.95% H2O, 0.1% FA (phase B), and the linear gradient was between 5% and 50% buffer (80% ACN, 20% H2O) for 80 minutes at a flow rate of 300 nL per minute. The mass spectrometer was operated in the data-dependent mode. A full scan survey MS experiment (m/z range from 400 to 2000, automatic gain control target 5E5 ions, resolution at 400 m/z was 100,000) was acquired from the mass spectrometer, and the five most abundant ions detected in the full scan were analyzed by MS/MS scan events (automatic gain control target IE4 ions, maximum ion accumulation time 200 ms). The normalized collision energy was 35%. The data acquired from the mass spectrometer were searched by Mascot against the database IPL_Human (V3.53). The Mascot search parameters were as follows: type of search, MS/MS ion search; mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ± 1.5 Da; fragment mass tolerance, ± 0.8 Da; and max missed cleavages, 2.

**Western Blot Analysis**

The proteins were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes at 200 mA at room temperature for 1 hour (2 hours for HMW proteins). The membranes were incubated with a monoclonal αA-crystallin antibody (1:1000; ab78439, Abcam, Cambridge, UK) and a monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:1000; ab8245, Abcam). The membranes were then incubated with an alkaline phosphatase-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Paso Robles, CA) at room temperature for 1 hour. The resulting bands on the immunoblots were visualized using a BCIP/NBT kit (Sigma, St. Louis, MO).

**Statistical Analysis**

Statistical analysis was performed by Student’s t-test and one-way ANOVA (DeCyder 6.5 software; GE Health Care) (2-D DIGE) and SPSS version 17.0 software (SPSS Inc, Chicago, IL), and P < 0.05 was considered to be statistically significant. The band intensity from the SDS-PAGE and Western blot experiments was quantified with image analysis software (ImageQuant TL; Amersham Biosciences) before statistical analysis.

**RESULTS**

**Differential Analysis of Protein Levels and Spot Identification**

To investigate the changes in ARNC lenses, 2-D DIGE was performed, with a 1.5-fold or greater difference in the amount of protein and a t-test score < 0.05 considered to be significant. A total of 39 protein spots were significantly different between normal (N) and ARNC (M) lenses (Fig. 1), and each was less abundant in the ARNC lenses compared with the normal ones. The peptides representing these spots were analyzed by MALDI-TOF mass spectrometry, and 36 spots were successfully identified (Table 2). Spot 2 was not identified by mass spectrometry because of low protein concentration, and identifications of spots 17 and 23 were not confident (without a statistically significant [P < 0.05] protein score). Based on these analyses, nine unique proteins were different levels between normal and ARNC lenses: αA-crystallin (1 spot), βA-crystallin (2 spots), βA4-crystallin (5 spots), βA4-crystallin (7 spots), βB1-crystallin (5 spots), γD-crystallin (3 spots), putative uncharacterized protein DKKFZp434A0627 (Crygs family; 3 spots), GAPDH (5 spots), and retinal dehydrogenase 1 (5 spots). For each unique protein, some of the spots were examined close up and three-dimensionally (Fig. 2). In the reverse-labeling experiment, we detected the same differences: low protein levels of the 39 spots in M compared with N (data not shown). Standardized log abundance was used to analyze changes in different groups (Fig. 3), which suggested that all nine unique proteins were less abundant in the ARNC lenses (A-G and M) than in the controls (N). The abundance of six unique proteins (αA-, βA3-, βA4-, βB1-, γD-crystallin, and DKKFZp434A0627) tended to decrease as the cataract grade increased (from grade II to IV). αB-crystallin and two enzymes (GAPDH and retinal dehydrogenase 1) did not show such a tendency. The abundance of these spots corresponding to the three proteins was very low in all cataract groups (data not shown). In addition, there was a high-intensity fluorescent band at the top of the gels (>200 kDa) in the lanes containing the ARNC samples but not in the lanes containing the normal samples. Because of their HMW, the proteins at the top of the gels could not be separated well using 2-DE. These bands may have been HMW aggregates of proteins with low molecular weights. To confirm this possibility, SDS-PAGE and LC-MS/MS analyses were performed.

**SDS-PAGE and LC-MS/MS Analyses**

After SDS-PAGE, the gel was imaged and analyzed with ImageQuant TL software (Fig. 4). In the normal lens nuclei (Lane N), most proteins were 15 to 30 kDa. In the ARNC lens nuclei (Lanes F, G, M, A-D, and E), the band intensity at ~20 kDa visibly (P < 0.05) decreased, but it significantly (P < 0.01) increased at >200 kDa when compared with the normal lens nuclei. As the grade of the cataract increased, the intensity levels of HMW proteins increased. In the grade IV ARNC lens nuclear sample (E), many proteins were found at the top of the gel. In contrast, the distribution of proteins in the grade II ARNC samples (F and G) was more similar to that of the normal samples (N). An intensity analysis of the SDS-PAGE images confirmed these intensity differences (Fig. 4B).

To determine whether the HMW proteins in the ARNC nucleus had some homology with the ~20 kDa proteins in the
Table 2. Proteins Identified by MALDI-TOF and Tandem TOF/TOF Mass Spectrometry

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<th>Pep. Count</th>
<th>MW (kDa)/pl</th>
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* Protein Mascot score obtained by MALDI-TOF and tandem TOF/TOF mass spectrometry identification; a score >60 indicates that the protein identification can be considered successful.
† Number of peptides matched.
‡ Theoretical molecular mass and pI calculated from the mature protein and amino acid sequence, respectively.
§ Average spot volume ratio between N (homogeneous mixture of seven normal lens nuclear extracts) and M (homogeneous mixture of seven ARNC lens nuclear extracts).
¶ Not identified by mass spectrometry because of low protein concentration.
§§ Not confident identification; confident identification had a statistically significant (P < 0.05) protein score (>60); based on combined mass and mass/mass spectra and the best ion score (based on mass/mass spectra).

normal nucleus, LC-MS/MS analysis was performed on gel slices from the lane containing the normal samples (N; slice 1; band at ~20 kDa) and the lane containing the ARNC samples (M; slice 2; band at >200 kDa). Nine and 14 proteins were identified in slice 1 and slice 2, respectively. A Mascot search revealed that each protein had an associated exponentially modified protein abundance index (emPAI) that was proportional to the protein content in the mixture. The relative abundances of each identified protein in slice 1 and slice 2 were estimated based on the emPAI data. Slice 1 contained five main proteins (accounting for 97% of the total proteins): γC, γB, αB, αA, and γD-crystallin, all of which had a molecular weight of ~20 kDa (19.9–20.9 kDa). γB1- and βB2-crystallin (28 kDa and 23 kDa, respectively) fragments, γA-crystallin (20.9 kDa), and a ~23 kDa protein fragment were also identified in the slice. Ten crystallins, including the five main crystallins in slice 1, together with filensin, phakinin, carbonyl reductase 1, and a ~23 kDa protein, were identified in slice 2. βA4-crystallin (22.4 kDa) matched the same set of identified peptides from the ~23 kDa protein, so the ~23 kDa protein identified in the two slices may have been βA4-crystallin. The relative abundance of each protein in the HMW aggregates (slice 2) is shown in Figure 5A. Of all the components, αA-crystallin accounted for the highest fraction (19.1%).

The compositions of the ARNC pooled sample (M) and the normal pooled control (N) were also analyzed by LC-MS/MS, and the relative abundance of each component was estimated (Figs. 5B and 5C). Eighteen proteins, together with one αA-crystallin fragment (17 kDa) and one βB1-crystallin fragment (10 kDa), were successfully identified in sample M. All the components in sample M, except the βB1-crystallin fragment, were also identified in sample N. In addition, small amounts of βB3-crystallin and GAPDH were only identified in sample N. Overall composition pattern of solubilizable proteins was sim-
ilar between control and ARNC. The relative abundances of retinal dehydrogenase 1, αA-crystallin, and the 17-kDa fragment in sample M (0.14%, 5.3%, and 4.0%, respectively) were lower and statistically significant (P < 0.05) when compared with those measurements in sample N (0.5%, 8.9%, and 5.4%, respectively). The other proteins identified both in sample M and sample N showed similar relative abundances in the two samples. Comparing relative abundance of the same crystallin among sample N, sample M, and slice 2 revealed that the aggregation tendency of each crystallin varied (Fig. 5). αA-, βB-, γD-crystallin, and DKFZp434A0627 were more prone to aggregate than others. By contrast, βA3-, βA4-, and γC-crystallin were relatively not prone to aggregate. Considering the absolute abundance of each protein identified in slice 2, αA-crystallin was a critical component of the HMW aggregates. βB1- and γS-crystallin and DKFZp434A0627 were also important components.

Western Blot Analysis

To verify the 2-D DIGE and SDS-PAGE results, two proteins were analyzed by Western blot. GAPDH was selected because it is an important glycolytic enzyme, and αA-crystallin was selected based on its dual role, as described above. By Western blot, GAPDH was significantly (P < 0.01) decreased in all ARNC samples (F, G, M, A–D, and E), which was consistent with the DIGE and LC-MS/MS results (Fig. 6C). The level of intact αA-crystallin (20 kDa) was also decreased (P < 0.05) in the ARNC samples, with the smallest amount in sample E (Fig. 6B). The monoclonal anti-αA-crystallin antibody also detected HMW aggregates and the αA-crystallin fragment (~17 kDa) (Figs. 6A and 6B). The intensities of the bands containing the HMW aggregates, αA-crystallin fragment, and intact αA-crystallin and corresponding ratios were determined (Fig. 6). Most aggregates were found at the top of the resolving gel (12%), with some also in the stacking gel (4%) in samples E and M (Fig. 6A). The intensity of the HMW aggregate bands increased as the lenses progressed from normal to cataract grade IV ARNC. The band intensity of the HMW aggregates was compared with that of intact αA-crystallin (H/I) in the different samples (Fig. 6D), with the results suggesting that more αA-crystallin aggregates formed during ARNC genesis and development. The ratio of the band intensity of the αA-crystallin fragment to that of intact αA-crystallin (F/I) did not show significant (P > 0.05) differences in samples N (normal), F and G (grade II), A–D (grade III), and M, but it was highest in sample E (grade IV) due to the small amount of intact αA-crystallin.

DISCUSSION

The aims of the present study were to produce 2-DE proteome maps of total proteins in human lens nuclei, analyze changes in the abundance of individual proteins in ARNC lenses, and
determine the compositions of irreversible HMW aggregates in ARNC lenses. To solubilize the water-insoluble protein, including urea-insoluble proteins and membrane proteins, powerful deaggregating reagents and chaotropes were used in the lysis buffer. To ensure that the aggregates were non– disulfide-linked, the IPG strips were treated with 0.5% DTT and 4.5% iodoacetamide after the first dimension of isoelectric focusing (IEF) and before the second dimension. Therefore, the HMW aggregates found in the ARNC lenses were irreversible and may include cross-links, or they may be firmly entangled noncovalently.

Recently, Asomugha et al.26 found HMW aggregates (>35 kDa, <120 kDa) in a 69-year-old normal lens; however, the HMW aggregates were much smaller than those present in ARNC lens nuclei (>200 kDa) in the present study. Thus, the mechanisms of protein aggregation in ARNC lenses may be different from those in the aging process. 2-D DIGE was used in this study to compare crystallin abundance in the cortex with that in the nucleus of an aging lens. In the present study, this technology was used to identify different levels of proteins in a direct comparison of normal and ARNC lens nuclei. The 2-D DIGE technique provided a reliable display of proteomic differences between samples based on the multiplexing approach for accurate matching across gels.27 2-D DIGE analysis identified 36 spots that represented nine unique proteins that were less abundant in ARNC lens nuclei. Because of the relatively reasonable lens numbers, we have provided for the first time the common changes and tendencies of some proteins in cataract lenses with different grades (Fig. 3). A combination of SDS-PAGE and LC-MS/MS analysis was adopted to determine the compositions of the HMW aggregates, and the emPAI was used to estimate relative protein abundances in the aggregates, as described above. This was unique and different from previous reported findings in the literature because of their difficulties in protein isolation and the availability of accurate methods for the compositional analysis.

2-D DIGE and LC-MS/MS analyses were mutually complementary in studying crystallin changes. Each unique crystallin showed several spots in 2-DE maps, and the 2-D DIGE method identified which spots significantly decreased in ARNC samples. The LC-MS/MS analysis of samples M and N identified in total the abundance of each unique crystallin containing intact, modified, and aggregated fractions, and most of the identified crystallins showed similar relative abundances in the two samples (Figs. 5B and 5C). Thus, these crystallins (αB-, βA3-, βA4-, βB1-, γD-crystallin, and DKFZp434A0627) decreased (2-D DIGE) due to aggregation and/or modification but not lower expression. The compositions of HMW aggregates found in ARNC lenses were quantified, and modifications need to be further identified using accurate quantitative methods. The total abundance of αA-crystallin and the 17-kDa fragment decreased (LC-MS/MS) due to aggregation and/or modification but not lower expression. The compositions of HMW aggregates found in ARNC lenses were quantified, and modifications need to be further identified using accurate quantitative methods. The total abundance of αA-crystallin and the 17-kDa fragment decreased (LC-MS/MS) due to aggregation and/or modification but not lower expression. The compositions of HMW aggregates found in ARNC lenses were quantified, and modifications need to be further identified using accurate quantitative methods. The total abundance of αA-crystallin and the 17-kDa fragment decreased (LC-MS/MS) due to aggregation and/or modification but not lower expression. The compositions of HMW aggregates found in ARNC lenses were quantified, and modifications need to be further identified using accurate quantitative methods. The total abundance of αA-crystallin and the 17-kDa fragment decreased (LC-MS/MS) due to aggregation and/or modification but not lower expression. The compositions of HMW aggregates found in ARNC lenses were quantified, and modifications need to be further identified using accurate quantitative methods. The total abundance of αA-crystallin and the 17-kDa fragment decreased (LC-MS/MS) due to aggregation and/or modification but not lower expression. The compositions of HMW aggregates found in ARNC lenses were quantified, and modifications need to be further identified using accurate quantitative methods. The total abundance of αA-crystallin and the 17-kDa fragment decreased (LC-MS/MS) due to aggregation and/or modification but not lower expression. The compositions of HMW aggregates found in ARNC lenses were quantified, and modifications need to be further identified using accurate quantitative methods. The total abundance of αA-crystallin and the 17-kDa fragment decreased (LC-MS/MS) due to aggregation and/or modification but not lower expression. The compositions of HMW aggregates found in ARNC lenses were quantified, and modifications need to be further identified using accurate quantitative methods. The total abundance of αA-crystallin and the 17-kDa fragment decreased (LC-MS/MS) due to aggregation and/or modification but not lower expression. The compositions of HMW aggregates found in ARNC lenses were quantified, and modifications need to be further identified using accurate quantitative methods. The total abundance of αA-crystallin and the 17-kDa fragment decreased (LC-MS/MS) due to aggregation and/or modification but not lower expression. The compositions of HMW aggregates found in ARNC lenses were quantified, and modifications need to be further identified using accurate quantitative methods. The total abundance of αA-crystallin and the 17-kDa fragment decreased (LC-MS/MS) due to aggregation and/or modification but not lower expression. The compositions of HMW aggregates found in ARNC lenses were quantified, and modifications need to be further identified using accurate quantitative methods. The total abundance of αA-crystallin and the 17-kDa fragment decreased (LC-MS/MS) due to aggregation and/or modification but not lower expression.
was the most abundant protein in the HMW aggregates suggesting the possibility of cross-linking between αA-crystallin and other proteins. The band of HMW aggregates was not seen in the normal lens nuclei (Fig. 4A), indicating that little aggregation occurred or that the aggregation was reversible in deaggregating reagents. These observations were confirmed by Western blot analyses of αA-crystallin (Figs. 6A and 6B). In Wistar rats, the loss of αA-crystallin is observed in the aging lens (10, 16, 30, 90, 180, and 360 days), and selenite-induced cataract rat models lose more αA-crystallin compared with age-matched normal rats. This phenomenon may be similar in human lenses. In vitro, α-crystallins can protect β- and γ-crystallins and other proteins against denaturation and subsequent aggregation. The mechanism of this protection involves preferential binding of the partially denatured protein to a central region of an α-crystallin complex, and the substrate proteins of the α-crystallins are in a molten globule state. The α-crystallins may perform a similar function in vivo, binding to and protecting other crystallins against further denaturation.

The molecular weights of the aggregates found in ARNC lenses were over 200 kDa. As α-crystallins aggregate to form a large complex, they may lose their chaperone abilities, thereby leading to an increase in the number of β- and γ-crystallin aggregates. Based on a study of HMW aggregates from aged bovine lenses, Boyle and Takemoto proposed that very large aggregates of α-crystallin may lose their ability to bind partially denatured proteins.

Previous studies have shown that the levels of βB1-crystallin decrease in Wistar rats with increasing age and that there is a significant decrease of βB1-crystallin in selenite-induced cataractous rats, compared with age-matched normal rats. In the present study, 2-D DIGE revealed a significant decrease in the levels of βA3-, βA4-, and βB1-crystallin in ARNC lens nuclei (Table 2), and βB1-crystallin accounted for 14.1% of the HMW aggregates by LC-MS/MS. However, LC-MS/MS analysis revealed that βA3- and βA4-crystallin were relatively not prone to aggregate, which indicated that the two proteins may suffer many modifications that may lead to the significant decrease identified by 2-D DIGE. Many posttranslational modifications of human β-crystallins have been identified, including deamidations of all except βB3, truncation of βA3, βB1, and βA4, and oxidation of some methionines and tryptophans. Excessive accumulation of deamidated β-crystallins in vivo may disrupt normal protein-protein interactions, diminishing their stability and thus contributing to the accumulation of insoluble β-crystallins during aging and cataract development.

The present study showed that DKFZp434A0627 and γS-, γD-, γC-, and γB-crystallin were components of the HMW aggregates (Fig. 5A). The putative uncharacterized protein DKFZp434A0627 (CRYGS family) was for the first time identified in human lenses and shown in 2-DE maps. It was a 14.3-kDa protein and more prone to aggregation than most crystallins. β- and γ-crystallin contain a high percentage of glutamine

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933461///09/23/2017)

**FIGURE 4.** SDS-PAGE image and intensity analyses of different samples. (A) The proteins were resolved by noncontinuous SDS-PAGE, and the gels were stained with Coomassie brilliant blue and then scanned. The first lane on the left contains a molecular weight marker. Lanes N, F, G, M, A–D, and E respectively represent a mixture of seven normal lens nuclear extracts, an extract of an ARNC lens nucleus of grade II, an extract of an ARNC lens nucleus of grade II, a mixture of seven ARNC lens nuclear extracts, a mixture of four extracts of ARNC lens nuclei of grade III, and an extract of an ARNC lens nucleus of grade IV. Slices 1 and 2 were cut manually and digested with trypsin for LC-MS/MS analysis. (B) Intensity analysis of lanes N (normal), F (ARNC; grade II), A–D (ARNC; grade III), and E (ARNC; grade IV). The intensity peaks for each lane and the molecular weights corresponding to pixel position are shown.
and asparagine deamidation, methionine and tryptophan oxidation, and truncations. These modifications could cause the β- and γ-crystallin to partially unfold and subsequently form aggregates. The aggregation of γD, γC, and γS were suppressed when they were incubated with an αB-crystallin chaperone, although its chaperoning abilities become increasingly overwhelmed as early adulthood progresses to advancing age. Because more α-crystallin accumulates in the HMW aggregates in ARNC lens nuclei than in normal lens nuclei and because aggregated α-crystallin likely loses its chaperoning abilities, the remaining α-crystallin will probably be overwhelmed prematurely, thereby making damaged β- and γ-crystallin more prone to aggregation.

GAPDH, an important enzyme in the glycolysis pathway, was significantly decreased in ARNC lenses compared with normal controls. However, there was no trend toward decreased levels of GAPDH as cataract grade increased. The levels of GAPDH were considerably diminished in all ARNC samples, indicating that GAPDH loss occurred either before opacification formation or during the early stage of cataract development. Investigators have shown that GAPDH activity in the cortex and nucleus of cataractous lenses is much lower than in clear lenses. A decrease in the amount of GAPDH in opaque nuclei has not been shown in previous studies.

FIGURE 5. Protein composition and relative abundance of each protein in slice 2 (HMW aggregates), ARNC pooled sample (M), and normal pooled control (N). The protein composition of each mixture was determined using LC-MS/MS and a Mascot search, and the relative protein abundance was calculated on the basis of emPAI data for each protein in the mixture. The protein abundances are described in molar percentages based on the formula: protein abundance (molar %) = emPAI/Σ (emPAI) × 100. If the relative abundance of a protein was below 0.5%, the number was not shown. (A) Protein composition and relative abundance of the HMW aggregates. (B) Protein composition and relative abundance in sample M. (C) Protein composition and relative abundance in sample N.

FIGURE 6. Western blot analysis of αA-crystallin and GAPDH levels in the ARNC lens nuclei compared with those in the normal lens nuclei. The Western blot experiments were repeated three times, and this figure represents once. The level of statistical significance has been shown in Results. The intensities of the immunoreactive bands (A and B) were estimated using ImageQuant TL software and are indicated below the respective lanes on the images (A, B, and C). Molecular weights are given to the right of the images. (A) Image of HMW aggregates (≥200 kDa) of αA-crystallin. The stacking gel (4%) is shown on the top, and the resolving gel (12%) is shown on the bottom. (B) Image of intact αA-crystallin (20 kDa) and αA-crystallin fragment (17 kDa). (C) Image of the GAPDH levels. (D) Profile of the ratio (band intensity) in different lanes. ▲ represents the ratio of HMW aggregates to intact αA-crystallin (H/I); ▼ represents the ratio of αA-crystallin fragment to intact αA-crystallin (F/I).
The lens is an avascular organ with a low concentration of oxygen; thus, most energy (adenosine triphosphate; ATP) for metabolism is produced through the glycolytic pathway, of which GAPDH is an important component. Low levels and/or low activity of GAPDH lead to inadequate ATP, which may reduce the chaperone function of α-crystallin. This possibility is supported by the findings of Biswas and Das, 43 who showed that ATP binding induced structural changes and enhanced chaperone function, even though α-crystallin does not require ATP hydrolysis for its chaperone function.

Similar to GAPDH, retinal dehydrogenase 1 was significantly decreased in all ARNC samples, including F and G (Grade II, mild opacity). To our knowledge, this change has been shown for the first time. In Aldh1a1(−/−) knock-out mice, lens opacification occurred later in life. 44 Retinal dehydrogenase 1 may protect the lens against cataract formation by detoxifying aldehydic products of lipid peroxidation in both the cornea and lens. 44 Further support for this mechanism is provided by the fact that the antimalarial drug chloroquine, which binds and inhibits retinal dehydrogenase 1, 5 induces cataracts in rats. 46 In human lenses, retinal dehydrogenase 1 may play such a protective role through the referred mechanism.

Asomugha et al. have shown that filensin and phakinin are present in the ∼100 kDa aggregates from an aging lens with no opacity. 20 In the ARNC lens nuclei (M) in the present study, filensin and phakinin were present in much larger aggregates (>200 kDa), which may be a vital reason for opacification in the nucleus. Previous studies have shown an association of α-crystallin with intermediate filaments and microtubules. 48–50 Its coexistence with considerable crystallins indicates that there may be cross-linking between them. Immunoprecipitation of native bovine αB-crystallin from heat-shocked lens cell homogenates resulted in the coprecipitation of filensin and phakinin. 51 Further, α-crystallin showed coassembly with phakinin and filensin in vitro studies, 52 and cross-linking between fragments of α, β, and γ-crystallin and the two beaded filament proteins has been shown in human lenses. 15

To our knowledge, the presence of the carbonyl reductase 1 in these aggregates is a novel finding. Carboxyl reductase is a cytosolic, monomeric oxidoreductase, and the role of carboxyl reductase in maintaining lens transparency remains unknown. The enzyme catalyzes the reduced form of nicotinamide adenine dinucleotide phosphate-dependent reduction of a great variety of carbonyl compounds. 53 Reactive carboxyls can promote oxidative stress, the products of which may be an important initiating factor in cellular damage in age-related processes. Oxidative stress has been implicated as a risk factor for age-related cataract. 54 An H2O2-induced cataract model has shown that oxidative changes precede a cascade of events before cataract formation, starting with protein disulfide cross-linking, protein solubility loss, and HMW protein aggregation. 55 Our results showing the aggregates containing carbonyl reductase 1 during cataract development suggest that multiple crystallins may be more vulnerable to oxidative stress because of diminished enzyme activity. The possible mechanisms need further investigation.

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References


