The Common Modification in αA-Crystallin in the Lens, N101D, Is Associated with Increased Opacity in a Mouse Model*

Received for publication, May 26, 2010, and in revised form, January 10, 2011 Published JBC Papers in Press, January 18, 2011, DOI 10.1074/jbc.M110.148627

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To elucidate the morphological and cellular changes due to introduction of a charge during development and the possible mechanism that underlies cataract development in humans as a consequence of an additional charge, we generated a transgenic mouse model mimicking deamidation of Asn at position 101. The mouse model expresses a human αA-crystallin gene in which Asn-101 was replaced with Asp, which is referred to as αAN101D-transgene and is considered to be “deamidated” in this study. Mice expressing αAN101D-transgene are referred to here CRYAA_N101D mice. All of the lines showed the expression of αAN101D-transgene. Compared with the lenses of mice expressing wild-type (WT) αA-transgene (referred to as CRYAA_WT mice), the lenses of CRYAA_N101D mice showed (a) altered αA-crystallin membrane protein (aquaporin-0 (AQP0), a specific lens membrane protein) interaction, (b) extracellular spaces between outer cortical fiber cells, (c) attenuated denucleation during confocal microscopic examination, (d) disrupted normal fiber cell organization and structure during scanning electron microscopic examination, (e) distorted posterior suture lines by bright field microscopy, and (f) development of a mild anterior lens opacity in the superior cortical region during the optical coherence tomography scan analysis. Relative to lenses with WT αA-crystallin, the lenses containing the deamidated αA-crystallin also showed an aggregation of αA-crystallin and a higher level of water-insoluble proteins, suggesting that the morphological and cellular changes in these lenses are due to the N101D mutation. This study provides evidence for the first time that expression of deamidated αA-crystallin caused disruption of fiber cell structural integrity, protein aggregation, insolubilization, and mild cortical lens opacity.

The ocular lens has a unique cellular architecture consisting of a single layer of cuboidal epithelial cells, which divide and differentiate at the equator into fiber cells (1). The fiber cells elongate, and they synthesize fiber cell-specific proteins, such as AQP0 (aquaporin-0)/MIP (main intrinsic protein), cytoskeletal proteins, and crystallins (1-4). As the new fiber cells are laid down at the lens equator, the older fiber cells are pushed toward the lens core and simultaneously lose their nuclei and organelles while exhibiting very little protein turnover. Among the three classes of the vertebrate lens crystallins (α-, β-, and γ-crystallins), α-crystallins are composed of two primary gene products, A and B, known as αA- and αB-crystallins, that show ~60% amino acid homology and constitute up to 50% of the total lens proteins. Both αA- and αB-crystallins belong to a family of small heat shock proteins with “chaperone” activity (5, 6). These crystallins are constitutively expressed in both lens epithelial and fiber cells (7) and undergo numerous age-related post-translational modifications (PTMs)2 that lead to their unfolding, aggregation, and insolubilization. These PTMs eventually lead to accumulation of damaged crystallins in the lens (8-11), which upon aggregation and cross-linking, precipitate and are believed to cause lens opacity. The PTMs also disturb the short range order of crystallins (12), which is dependent on their specific interactions. The fiber cells contain several intermediate filaments, which include vimentin, phakinin (CP49), and filensin (13), and these have been found to associate with membranes (14). α-Crystallins have also been reported to be associated with cytoskeletal proteins (actin, microtubules, and intermediate filaments) (15, 16). A FRET photobleaching study showed the αB R120G mutant facilitated aggregation of vimentin by altering protein-protein interactions (17). A recent study with the WT/R49C<sup>neo</sup> mouse model showed disruption of normal fiber cell organization and their structure in the deep cortex (18). These reports suggest that cytoskeletal proteins and crystallins interact with membranes and play a structural role in maintaining the lens transparency.

Several PTMs in crystallins have been reported that cause their structural and functional instability (19, 20). Deamidation has been identified as the most prevalent among PTMs in aging human lenses (10, 20). Deamidation of asparagine to aspartic acid and/or glutamine to glutamic acid adds an additional negative charge, which substantially alters structural and functional properties of αA- and αB-crystallins (21, 22). The site of deamidation along with the three-dimensional structure near the amide group and protein secondary structures (such as α-helices or random coils) also affects the deamidation rate in vivo (23). Our previous report showed that the deamidation of Asn-101 in αA has relatively more deleterious effects on its structure and function than deamidation of Asn-123 (21). However, while studying the effects of deamidation along with truncation of N and/or C termini on αA-crystallin, it was the

2 The abbreviations used are: PTM, post-translational modification; Tg, transgenic; WS, water-soluble; WI, water-insoluble; OCT, optical coherence tomography; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycoltetraacetic acid; MALS, multiangle light scattering; SDS, sodium dodecyl sulfate.
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Asn-123 residue that was crucial in maintaining the chaperone function (24). Therefore, deamidation alone or in combination with other PTMs, such as truncation, exhibited different effects on structure and function of αA-crystallin during in vitro studies. These deamidations also alter the subunit exchange rate between αA- and αB-crystallins, which is believed to influence their chaperone function (21–24).

Lens transparency is dependent on the interactions among crystallins, maintenance of cellular homeostasis, and proper cellular ionic concentrations. Mutations in the fiber cell-specific proteins, such as main intrinsic protein (aquaporin-0) and connexins, lead to lens opacity, which is mediated by activation of lens-specific calpain or inhibition of the Na,K-ATPase (25, 26). As stated above, α-crystallin is known to associate with lens fiber cell membranes in vitro, and the extent of binding increases with both age and diseased conditions (27, 28). Because deamidation of crystallins alters their structure and function, it is likely that interaction of the deamidated crystallins with other crystallins, membranes, and fiber cell-specific protein(s) are also affected and might lead to morphological changes and abnormalities in fiber cells (18, 28). Despite these reports, the in vivo effects of deamidated αA-crystallin are presently not known. It is unclear whether the deamidated αA-crystallin in vivo would affect (a) αA interaction with membrane, (b) αA interaction with other crystallins and cytoskeletal proteins such as actin, tubulin or intermediate filaments, (c) differentiation of epithelial cells into fiber cells, and (d) lens opacification.

Deamidation of αA-Asn-101 was reported to be 50% (i.e. ~45% during 0–39 years and 5% during 30–68 years of age) in human lenses (29). Despite such high prevalence of deamidated αA Asn-101-crystallin in human lenses, its potential effects on structure and function of the crystallin have only been studied in vitro. Because of eminent deleterious effects of αAN101D on its structure and function, the present study was undertaken to determine phenotypic changes in fiber cells and altered crystallin solubility in a transgenic mouse model expressing αAN101D-transgene. This study identified, for the first time, comparative changes in lenses of CRYAA_{N101D} and CRYAA_{WT} mice to provide insight into how “deamidation” of αA Asn-101 to an Asp residue influences its interaction with membrane proteins, protein solubility, and lens opacity. Our results show that compared with wild type (WT), the introduction of a charge during development in αA-crystallin showed altered interaction with fiber cell membrane, altered packing of fiber cells, persistence of nuclei in the deeper cortical region, increased water insolubilization of the crystallin, and mild cortical lens opacity.

EXPERIMENTAL PROCEDURES

Materials

The restriction endonucleases, the molecular weight protein markers, and DNA markers were purchased from Amersham Biosciences and Promega (Madison, WI), respectively. Sequencing primers and other primers used in the study were obtained from Invitrogen. Unless stated otherwise, the molecular biology-grade chemicals were purchased from Sigma-Aldrich or Fisher. Site-specific polyclonal antibodies against the desired αA-crystallin epitopes were raised in rabbits in our laboratory by using a standard procedure as previously described (30). The polyclonal anti-human aquaporin-0 (AQP0) antibody, raised in rabbits, was purchased from Alpha Diagnostics (San Antonio, TX), and mouse monoclonal anti-His antibody was obtained from Novagen (Madison, WI).

Generation of Transgenic Mice

The mouse model that expresses a human αA-crystallin gene in which Asn-101 was replaced with Asp is referred to as αAN101D-transgene. This model has been considered to be “deamidated” in this study, and the mice expressing αAN101D-transgene are referred to here as CRYAA_{N101D} mice. The plasmid pIVS2, containing the mouse 409-bp αA-crystallin promoter and human WT αA-crystallin cDNA tagged with a His7 tag epitope at its N terminus (obtained from Dr. Mark Petrasch, University of Colorado, Denver, CO) (33) was used as a template to produce the deamidated construct. The αAN101D-transgene was generated by PCR amplification with mutated primers using a site-directed mutagenesis kit (Stratagene): sense primer, 5’-ATCCACGGAAAGACGACGAGGCCA-GGACGACC-3’ and the antisense primer 5’-GCGGCTCTGGGCCTGTCGTGCTTTCCTGGAT-3’. The resultant PCR amplicon was cloned into pIVS2 at the unique BamHI site. The position and integrity of the αAN101D-transgene was confirmed by digestion with restriction enzymes (Sphl and Apal) and DNA sequence analyses at the DNA Core Facility of the University of Alabama at Birmingham. The vector sequence was removed by Smal and AccI digestion, and the purified linear transgene fragments were injected into fertilized BL6 cell embryos at the Transgenic Mouse Core Facility of the University of Alabama at Birmingham to obtain CRYAA_{N101D} mice. PCR-based genotyping was used to screen potential founders for the presence of αAN101D-transgene from genomic DNA extracted from tail biopsies using the Qiagen (Valencia, CA) DNaseasy tissue kit. Independent transgenic (Tg) mouse lines were established from transgenic founders using C57BL/6 mice (Harlan Laboratories, Indianapolis, IN). Two male transgenic mouse lines expressing WT αA-crystallin with a His7 tag epitope at its N terminus (referred as CRYAA_{WT}) were obtained from Dr. Mark Petrasch (strain 10694) (33). These were used for further breeding and expanding the colony. The positive mice (after PCR-based genotyping as described above) were used as a control. All procedures in this study were done as per the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Transcriptional activity of αAN101D-transgene was confirmed by RT-PCR. Total RNA from non-Tg, CRYAA_{WT}, and CRYAA_{N101D} mouse lenses was isolated using the Qiagen RNeasy Kit. cDNA was prepared using a kit from Promega (Madison, WI). A full-length transcript (525 bp) and a 250-bp fragment were amplified from oligo(dT)-primed cDNA using a pair of gene-specific primers in the αA-crystallin. The two
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primers contained a His\textsubscript{7} tag epitope that allowed distinction of human transgene product from endogenous mouse αA-crystallin. Primer sequences were as follows: upstream, 5′-ACA-TGGGACATCATCATCATCATCATGAGAC-3′; downstream, 5′-GAGGAAGATGACGAACTTGTCCCGG-3′ and 5′-TTAGGACGAGGGAGCCGAGGTGGG-3′.

**Preparation of Lens Homogenate**

Under a dissecting microscope, the lenses were dissected in PBS from eyes of non-Tg, CRYAA\textsubscript{WT}, and CRYAA\textsubscript{N101D} mice and homogenized in buffer A (5 mM Tris-HCl, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 8.0, and protease inhibitor mixture (Roche Applied Science)), and centrifuged at 14,000 × g for 30 min at 4 °C to separate the water-soluble (WS) and water-insoluble (WI) protein fractions. The supernatants (soluble protein fractions) were collected, and the above steps of suspension in buffer A, centrifugation, and water-soluble/water-insoluble protein fraction recovery were repeated three times. The water-insoluble protein fraction (pellet) was solubilized in 5 mM Tris-HCl, pH 7.5, containing 4 M urea, 5 mM EDTA, and 5 mM EGTA. The protein concentration was measured by a Pierce kit using bovine serum albumin as a standard.

**Preparation of Lens Fiber Cell Membranes**

Lenses were recovered from non-Tg, CRYAA\textsubscript{WT}, and CRYAA\textsubscript{N101D} mice immediately after euthanization and homogenized in buffer A (5 mM Tris-HCl, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 8.0, and 0.01% bromphenol blue).

**Miscellaneous Methods**

**Lens Protein Analysis**—Assessment of expression levels of lens protein (α-, β-, and γ-crystallins) was carried out by HPLC using a size exclusion TSK G-4000 PW\textsubscript{XL} column coupled to an online UV detector, a dynamic multiangle laser light scattering detector, and a refractive index detector, Optilab-DSP (Wyatt Technology). The analysis also determined the absolute molar mass of the α-crystallin fraction in the WS-protein fraction of lenses from CRYAA\textsubscript{WT} and CRYAA\textsubscript{N101D} mice as described earlier (24). Aggregation of WT αA-crystallin and deamidated αA-crystallin was determined by Western blot analysis (32) using a monoclonal antibody to His tag epitope as described below.

**Western Blot Analysis**—SDS-PAGE analysis of lens proteins was carried out by the Laemmli method (31), and the Western blot analysis was by the method of Towbin et al. (32). The protein samples from WS, WI, and membrane protein fractions were separated on a 15% polyacrylamide gel by SDS-PAGE and transferred onto the PVDF membrane. After blocking the nonspecific sites with 5% bovine serum albumin (BSA) in PBS, the membrane was incubated with monoclonal antibodies against His\textsubscript{7} tag (in WT αA/αA101D) and polyclonal antibodies against AQP0 at 4 °C for 16 h. Immune complexes were detected using goat anti-mouse IgG or goat anti-rabbit IgG antibodies conjugated with Cy2 (green)/Cy5 (red) fluorophores and scanned with Typhoon Trio Imager at excitation 488/633 nm, respectively. The green and red fluorescence showed the presence of WT αA/αA101D-transgene and AQP0, respectively.

**Immunohistochemistry**—Eyes from non-Tg, CRYAA\textsubscript{WT}, and CRYAA\textsubscript{N101D} mice were enucleated from postnatal 1-, 3-, 5-, and 7-month-old mice and fixed overnight in 10% formalin/PBS at room temperature for routine histology. For immunostaining, deparaffinized sections were treated with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0), followed by 3% hydrogen peroxide treatment for 30 min to remove endogenous peroxidase wherever necessary. After blocking with 20% normal calf serum for 30 min, the sections were probed overnight at 4 °C with the desired primary antibody diluted into the blocking solution. The monoclonal antibodies against His\textsubscript{7} tag (in WT αA/αA101D) were used to detect the transgene, whereas a polyclonal antibody to AQP0 was used to analyze the fiber cell structure. Primary antibody labeling was detected using (a) a rabbit IgG ABC kit ( Vectastain Elite; Vector Laboratories, Inc., Burlingame, CA) and 3′,3′-diaminobenzidine as the peroxidase substrate and (b) goat anti-mouse or goat anti-rabbit antibodies conjugated with Alexa fluorophores (Invitrogen). Lens sections were counterstained with hematoxylin or Hoescht stain for nuclei staining. Negative controls were treated identically minus primary antibodies. Sections were coverslipped and photographed digitally using an Zeiss AxioPlan 2 microscope and Zeiss laser-scanning confocal microscope LSM 710 equipped with an Axiovert inverted microscope with ZEN 2008 software for each fluorophore.

**Gross Lens Morphology**—Mice were euthanized, and eyes from postnatal 1-, 3-, 5-, and 7-month-old non-Tg, CRYAA\textsubscript{WT}, and CRYAA\textsubscript{N101D} mice were enucleated. The lenses were removed and photographed with a digital camera (Nikon) attached to a dissecting microscope (Stemi 2000, Carl Zeiss Meditec, Jena, Germany) within 10–15 min of euthanization.

**Optical Coherence Tomography (OCT)**—Lens opacity was measured with an ultrahigh resolution spectral domain optical coherence tomography instrument (SDOCT, Biotigen, Durham, NC). Anterior segment imaging of each eye from CRYAA\textsubscript{N101D} and CRYAA\textsubscript{WT} mice was performed by dilating eyes with 1% tropicamide drops and anesthetizing with a subcutaneous injection (5.5 times body weight) of a mixture of 2.4 ml of 15% ketamine hydrochloride and 0.8 ml of 2% xylazine hydrochloride, which was diluted in 6.8 ml of sterile saline. Next, the mice were placed in the cylindrical holder and mounted on the positioning stage in front of the optical scanning 24-mm human telecentric probe, and each eye was imaged along the entire axial length. To minimize the anesthesia time, all of the preparations were done in advance, and a mock imaging was done using non-Tg mice. The imaging was completed within 4–5 min. Analogous to an ultrasound B-scan, SDOCT acquires multiple A-scans with a bandwidth of 130 nm centered at 840 nm (scan length, 10 mm; scan depth, 2.3 mm). The scan...
line was adjusted to a horizontal position bisecting the pupil. The area selected for lens density measurement encompassed the superior cortical, nuclear, and inferior cortical regions. The image processing and analysis were performed using ImageJ and Adobe Photoshop software. The pixel intensity in the area selected for lens cortical cataract density was calculated as the mean pixel intensity after adjusting the background noise at the anterior chamber.

Scanning Electron Microscopy (SEM)—The SEM analyses of lenses were performed at the Core Facility of the University of Alabama at Tuscaloosa. The eyes were enucleated from non-Tg, CRYAA<sub>WT</sub> and CRYAAN<sub>101D</sub> mice after euthanization. The tissues were then fixed overnight in 0.12 M sodium cacodylate, 2.25% glutaraldehyde, 1% paraformaldehyde at 4 °C. Next, lenses were excised and bisected along the optical axes, fixed in 2% (w/v) paraformaldehyde and 2% glutaraldehyde (v/v) in 0.1 M sodium cacodylate buffer, pH 7.2, at room temperature for 24 h. Samples were then washed several times with distilled water and postfixed in 1% (v/v) aqueous osmium tetroxide at room temperature for 1 h. Lens halves were dehydrated in an ascending ethanol series from 30 to 100%, followed by critical point drying in carbon dioxide in a Denton DCP-1 (Capovani Brothers Inc., Scotia, NY). Critical point-dried lens halves were secured on Pelco Carbon tabs mounted on aluminum stubs. Mounted specimens were then sputter-coated with a mix of gold and palladium and viewed on a Hitachi S-2500 scanning electron microscope at 5–10 kV.

RESULTS

CRYAA<sub>101D</sub> Transgenic Mice—to determine the effect of introduction of a charge during development (Asn-101 → Asp) in αA-crystallin in vivo, the expression of deamidated αA-crystallin was specifically targeted in the lens fiber cells by employing a mouse αA-crystallin promoter fragment, which was previously used to induce lens fiber cell-specific expression in a transgenic mouse model by Dr. Mark Petrash (33). Because the anti-αA antibodies could not distinguish between mouse endogenous αA-crystallin and transgenically expressed αA-crystallin, an in-frame His<sub>6</sub> epitope tag was included at the N terminus of the coding region of human αA-crystallin (Fig. 1A). A similar approach was previously taken by Petrash and co-workers (33) to examine the effects of a congenital mutation (R116C) of the human lens in transgenic mice. The DNA sequencing confirmed the point mutation of Asn-101 to Asp in the human lens in transgenic mice. The DNA from non-Tg littermates using either set of primers (as described under “Experimental Procedures”).

Transgenic protein expression in founder lines of CRYAA<sub>101D</sub> (i.e. lines 1.1, 1.B2, 3.42, and 2.21) as well as CRYAA<sub>WT</sub> lines (i.e. 2.1 and 2.2) was assessed by the multiplex Western blot method and immunohistochemical analysis (Fig. 1, D and E). In the lens homogenates, a major band of ~20 kDa (including a strong doublet that did not separate on a 15% polyacrylamide gel during SDS-PAGE) was detected as an immunoreactive species with anti-His tag antibody (Fig. 1D, left). This band along with a cleaved form of αA or its degradation products also exhibited immunoreactivity with anti-αA crystallin antibody in transgenic mice homogenates (Fig. 1D, right). However, the levels of the immunoreactivity with both antibodies were variable in lens homogenates prepared from different lines. The -fold difference in the expression among different lines was computed using the one-dimensional gel analysis module of ImageQuant TL 7.0 (GE Biosciences). CRYAAN<sub>101D</sub> line 1.B2 showed relatively greater (~4-fold) expression of αA compared with the other three transgenic lines. Lens homogenates from non-Tg mice showed no detectable binding of anti-His tag antibody to proteins (~20 kDa; Fig. 1D, left, lane 1). Immunohistochemical staining of eye sections from CRYAA<sub>WT</sub> and CRYAAN<sub>101D</sub> mice further confirmed the above Western blot results and showed variable immunostaining intensity (Fig. 1E). A representative figure (Fig. 1E, left, at ×10 and ×40 magnifications) shows no immunoreactivity of anti-His antibody to lenses from non-Tg mice compared with CRYAA<sub>WT</sub> and CRYAAN<sub>101D</sub> mice. Together, these results showed comparable expression levels of WT and αAN101D-transgenic protein, suggesting that the difference in phenotypical and cellular changes observed in CRYAA<sub>WT</sub> and CRYAAN<sub>101D</sub> mice are due to the N101D mutation. CRYAAN<sub>101D</sub> line 1.B2 and CRYAA<sub>WT</sub> line 2.1 showed relatively high transgene expression during Western blot and immunohistochemical analyses compared with other lines (Fig. 1, D and E); these two lines were selected and further used for a comparative examination of any lens abnormality, morphological changes in fiber cells, and levels of insoluble proteins.

Expression of αAN101D Protein in Lens Fiber Cells Results in Mild Cortical Opacity—Bright field microscopy using Zeiss Stemi 2000 dissection microscope was used to obtain the lens micrographs from non-Tg, CRYAA<sub>WT</sub>, and CRYAAN<sub>101D</sub> mice. This method was previously used to determine lens opacity and suture defects from a congenital mutation (R116C) of the human lens in transgenic mice (33). The lens suture abnormalities were determined at ages of 1 month (Fig. 2), 3 months, 5 months, and 7 months in CRYAA<sub>101D</sub> line 1.B2 and CRYAA<sub>WT</sub> line 2.1. Lens opacity was further analyzed by the OCT method as described under “Experimental Procedures” (Fig. 3). Although an outer cortical haze was observed in all
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FIGURE 1. Generation of CRYAA\textsubscript{N101D} mice. A, deamidated \(\alpha\)-a-crystallin transgene construct for microinjection. Shown is a diagram of the transgene construct containing the \(\alpha\)-A-crystallin promoter, human \(\beta\)-globin intervening sequence, the coding region of deamidated \(\alpha\) at residue 101 (\(\alpha\)AN101D), an in-frame epitope tag (His\(_7\), 7X-His) at the N terminus of \(\alpha\)-A-crystallin, untranslated sequences (UTT), and a polyadenylation signal. The plasmid pIVS2, containing the mouse 409-bp \(\alpha\)-A-crystallin promoter and human WT \(\alpha\)-A-crystallin cDNA tagged with a His\(_7\) tag epitope at its N terminus was obtained from Dr. Mark Petrash (33). B, detection of the transgene by PCR-based genotyping in independent founder transgenic mice. Results from 22 potential founder mice (lanes 2–24) are shown. Four founders showed positive strong bands (lanes 6, 8, 15, and 21), whereas one showed a relatively weak band (lane 18). The PCR conditions were standardized by using the human AN101D-crystallin construct at a single copy level per genome (lane 26; a positive control). C, RT-PCR analysis of transgene expression in CRYAA\textsubscript{N101D} and CRYAA\textsubscript{WT} lines. Transcriptional activity of the transgene was confirmed by RT-PCR. Results from four CRYAA\textsubscript{N101D} lines (1.1, 1.B2, 3.42, and 2.21) are shown. Full-length mRNA transcript was amplified from oligo(dT)-primed cDNA using a pair of gene-specific primers in the \(\alpha\)-A-crystallin. The primers contained His\(_7\) tag epitope to distinguish human transgene product (\(\alpha\)AN101D) from endogenous mouse \(\alpha\)-A-crystallin. RT-PCR showed no amplified product from RNA extracts of non-transgenic pups (lane 2). The CRYAA\textsubscript{WT} mouse was used as a control and was a gift from Dr. Mark Petrash as described under “Experimental Procedures” (33). D, Western blot analysis of lens protein preparations (identical lanes in both left and right panels). Lane 1, non-Tg (NT); lane 2, CRYAA\textsubscript{WT} line 1.1; lane 3, CRYAA\textsubscript{WT} line 1.2; lane 4, CRYAA\textsubscript{WT} line 1.1.B2; lane 5, CRYAA\textsubscript{WT} line 1.2.B; lane 6, CRYAA\textsubscript{WT} line 1.1; lane 7, CRYAA\textsubscript{WT} line 3.42. E, immunohistochemical analysis of paraffin-embedded eye sections from different lines of CRYAA\textsubscript{WT} and CRYAA\textsubscript{N101D}. CRYAA\textsubscript{N101D} line 1.B2 and CRYAA\textsubscript{WT} line 2.1 showed relatively higher transgene expression during Western blot and immunohistochemical analyses of tissue sections compared with other lines; these two lines were selectively examined further for morphological changes in fiber cells, lens abnormality, and levels of insoluble proteins. Note that lens preparation (top two panels on left) from the non-transgenic mice did not show any immunoreactivity with an anti-His tag antibody.

FIGURE 2. Differences in suture lines in lenses of CRYAA\textsubscript{WT} and CRYAA\textsubscript{N101D} Mice. Bright field micrographs of lenses from 1-month-old (1m) CRYAA\textsubscript{WT} line 2.1 (A) and CRYAA\textsubscript{N101D} line 1.B2 mice (B) showed differences in fiber cells, lens abnormality, and levels of insoluble proteins. Note that lens preparation (top two panels on left) from the non-transgenic mouse did not show any immunoreactivity with an anti-His tag antibody.

lenses, there were subtle differences between the lenses from CRYAA\textsubscript{WT} and CRYAA\textsubscript{N101D} mice (data not shown). The suture lines of lenses of CRYAA\textsubscript{N101D} mice at 1 month of age were distorted at the posterior pole (Fig. 2B, dotted arrow) and were clearly different compared with an inverted Y-shaped suture line seen in lenses of CRYAA\textsubscript{WT} mice (Fig. 2A, solid arrow). The lenses from the CRYAA\textsubscript{N101D} mice at 3, 5, and 7 months of age showed nondescript posterior suture lines compared with age-matched lenses of CRYAA\textsubscript{WT}, which did not show any such abnormality (data not shown). The 5-month-old lenses of CRYAA\textsubscript{N101D} mice also showed apparent fiber cell swelling in the outer cortex (data not shown). These results showed that apparently deamidation in \(\alpha\)-A-crystallin affected lenses of CRYAA\textsubscript{N101D} mice as early as 1 month of age.
OCT is a non-invasive imaging technology that employs light to create detailed images of cornea, lens, and retina; it was used to further confirm whether the lens haziness as observed above was due to lens opacity. The OCT scans of lenses from 1–7-month-old mice were determined. The OCT scans of different regions (i.e., superior cortical, nuclear, and inferior cortical) of lenses of CRYAAN101D line 1.B2 and CRYAAWT line 2.1 were determined. The lens opacity was observed beginning at 5 months of age in CRYAAN101D mice compared with CRYAAWT mice. The representative OCT images of the anterior and posterior segments from 7-month-old mice, including the cornea in both eyes of the above described mice, are shown in Fig. 3. In this study, multiple A-scans obtained from OCT assessed the cataract morphology and density at different meridians: inferior cortical, nucleus, and superior cortical. The 7-month-old lenses showed relatively more opacity in the superior cortical region (Fig. 3, H and K) as compared with the inferior cortical region (Fig. 3, I and L) and nuclear region (Fig. 3, G and J). The OCT intensities in the inferior cortical, nuclear, and superior cortical regions were 6, 8, and 8 in lenses of CRYAAN101D mice compared with 8 units in comparable age lenses of CRYAAWT mice. The OCT is a non-invasive imaging technology that employs light to create detailed images of cornea, lens, and retina; it was used to further confirm whether the lens haziness as observed above was due to lens opacity. The OCT scans of lenses from 1–7-month-old mice were determined. The OCT scans of different regions (i.e., superior cortical, nuclear, and inferior cortical) of lenses of CRYAAN101D line 1.B2 and CRYAAWT line 2.1 were determined. The lens opacity was observed beginning at 5 months of age in CRYAAN101D mice compared with CRYAAWT mice. 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The above histological changes in fiber cells of lenses from CRYAA\textsubscript{N101D} mice were further confirmed by immunohistochemical analysis, followed by confocal microscopic examination (Fig. 5). During the immunohistochemical analysis, the axial and equatorial lens sections were immunoreacted with the anti-His tag monoclonal (green fluorescence) and anti-AQP0 polyclonal (red fluorescence) antibodies and viewed under a confocal microscope. At a low magnification (10×), the equatorial and axial sections of lenses from 1-, 3-, 5-, and 7-month-old CRYAA\textsubscript{N101D} mice were compared with age-matched lenses of CRYAA\textsubscript{WT} mice. An examination of 1-month-old equatorial sections from CRYAA\textsubscript{N101D} and CRYAA\textsubscript{WT} mice lenses showed no morphological difference except a decrease in the lens size in the former (data not shown). Similarly, the axial sections of 3-month-old lenses did not show any notable morphological changes (data not shown). CRYAA\textsubscript{N101D} mice began to show an alteration in fiber cell shape and alignment at the bow region at an age of 5 months (data not shown); however, these differences were more pronounced at 7–9 months of age in the lenses of CRYAA\textsubscript{N101D} mice compared with WT lenses. Compared with the WT control (Fig. 5, A and B), the lenses from 7-month-old CRYAA\textsubscript{N101D} mice showed disorganization of radial fiber cell columns. Severe degeneration of outer cortical fiber cells in lenses of CRYAA\textsubscript{N101D} mice (Fig. 5D, arrow) compared with lenses from CRYAA\textsubscript{WT} mice (Fig. 5C, arrow) was also observed. Immunostaining with antibodies against membrane protein (AQP0, red fluorescence) confirmed the irregular shaped fiber cells in outer cortex, altered membrane morphology, and misaligned fiber cell arrays in lenses of 7-month-old CRYAA\textsubscript{N101D} mice (Fig. 5D) compared with the lenses of CRYAA\textsubscript{WT} mice (Fig. 5B). At 7 months of age, the lenses from CRYAA\textsubscript{N101D} mice also exhibited enlarged extracellular spaces between outer cortical fiber cells and within the fiber cell arrays (Fig. 5D), irregularly shaped fiber cells interspersed in the outer cortical region compared with typical patterns of radial organization of fiber cells in 7-month-old WT lenses (Fig. 5B). The extracellular space fiber cells were not vacuoles because, as described below, the SEM analysis observed that membranes encompassed these large objects. Further, the nuclear staining (blue fluorescence) with Hoescht stain again confirmed the
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(A) Equatorial

10X-7m WT

(C) 10X-7m N101D

(E) 40X-7m WT

(B) Axial

10X-7m WT

(D) 10X-7m N101D

(F) 40X-7m N101D
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To ascertain if binding of αAN101D-transgene to fiber cell membranes was altered, the confocal microscopic images of lenses at 7 months of age were analyzed at ×40 magnification (Fig. 5, E and F). Upon immunoreaction of comparable sections of lenses of CRYAAN_{101D} mice and CRYAA_{WT} mice with anti-AQP0 (red fluorescence) showing a membrane protein and anti-His (green fluorescence due to αA) antibodies, a relatively increased yellow fluorescence in the outer cortical region (representing a colocalization of αA and AQP0) was seen in lenses of transgenic mice. In lenses of CRYAAN_{101D} mice, αA was detected all over the fiber cells (Fig. 5F), unlike discrete membrane-bound fiber cells in the CRYAA_{WT} (Fig. 5E), which suggested significant alterations in interaction between αAN101D-transgene and AQP0 in the former. Together, the results suggested that deamidation of the αA Asn-101 to an Asp residue disrupted the symmetrical array alignment of fiber cells following their differentiation from epithelial cells at the bow region and also altered the interaction between αA and AQP0.

SEM analysis further confirmed the results of immunohistochemical-confocal microscopic analyses. Because the above results suggested distinct morphological differences in fiber cells of CRYAAN_{101D} compared with CRYAA_{WT} mice at an age of 5 months and older, SEM selectively analyzed lenses of 5-, 7-, and 9-month-old mice. Representative SEM images from lenses of lenses at 7 months of age were analyzed at ×1000 magnification. Immunostaining with antibodies against AQP0 (a membrane-specific protein; red fluorescence) and His tag (green fluorescence due to αA) antibodies, showed irregularly shaped fiber cells in the outer cortex, altered membrane morphology, and misaligned fiber cell arrays in lenses of CRYAAN_{101D} mice compared with CRYAA_{WT} mice. Scale bar, 50 μm. E and F, Immunohistochemical localization of AQP0 and αA-crystallin in lenses of CRYAA_{WT} and CRYAAN_{101D} mice. Tissue sections immunoreacted with anti-AQP0 (red fluorescence showing the membrane protein) and anti-His (green fluorescence due to αA) antibodies showed increased association of αAN101D-crystallin with AQP0 in CRYAA_{WT} mice compared with lenses of CRYAA_{WT} mice. Note a greater association of αAN101D-transgene with AQP0 as represented by their colocalization (yellow fluorescence) in lens sections of CRYAA_{101D} mice (F) compared with CRYAA_{WT} mice (E). This suggested a relatively greater binding of deamidated αA compared with WT αA with fiber cell membranes. Scale bar, 20 μm.

FIGURE 6. SEM of CRYAA_{WT} and CRYAA_{N101D} mouse lenses. The SEM images of lenses from 7-month-old CRYAA_{WT} mice are shown in A and C, and lenses from CRYAAN_{101D} mice are shown in B and D. The images were recorded at a magnification of ×500 and ×1000. The regions that were magnified to ×1000 are represented by a box with white outline. The regular parallel arrays and ball-and-socket interdigitations within the parallel arrayed fibers in CRYAA_{WT} mice were observed in the outer cortical region (Ai). In contrast, the lenses of CRYAAN_{101D} mice (Bi) exhibited abnormal membrane structures resulting in irregular interlocking junctions represented by disorganized ball-and-socket interdigitations in tightly packed fiber cell arrays. The ordered formation and ball-and-socket interdigitations within the parallel arrayed fibers from CRYAA_{WT} and disorganization in CRYAAN_{101D} mouse lenses were clearly evident in C and D, respectively, which were captured at higher power with the same magnification (Di). Distinct differences in the widths of fiber cells of CRYAA_{WT} (Ci) and CRYAAN_{101D} (Di) mouse lenses were also evident in these images. Compaction of concentric layers of elongated fiber cells and irregular interdigitation in the inner cortical region is shown in CRYAA_{101D} (F) compared with CRYAA_{WT} (F) mouse lenses. Scale bar, 60–300 μm.
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from 7- and 9-month-old CRYAA_{WT} and CRYAA_{N101D} mice are shown in Fig. 6. Because of the lack of exact orientation regarding the equatorial regions of the lenses during SEM analysis, one of the four quadrants of a lens of a 7-month-old CRYAA_{WT} mouse and those of the CRYAA_{N101D} mice were initially analyzed at ×100 magnification (data not shown). Next, the selected quadrants of the lenses from CRYAA_{WT} and CRYAA_{N101D} mice were analyzed at ×500 and ×1000 magnifications, which showed distinct differences in morphology of fiber cells. The selected region of the lens quadrant from CRYAA_{WT} at ×500 is shown in Fig. 6A, which at ×1000 magnification exhibited straight radial fiber cell columns (Fig. 6A). In contrast, comparable magnifications of the selected region in CRYAA_{N101D} lenses (Fig. 6B) at ×500 (Fig. 6B) and ×1000 (Fig. 6B) showed disorganized fiber cells in parallel arrays. Further, SEM analysis also showed that the characteristic ball-and-socket interdigitations of fiber cells were intact in the CRYAA_{WT} lens but were disorganized in the CRYAA_{N101D} lens. In order to emphasize the ordered formation and ball-and-socket interdigitations within the parallel arrayed fiber cells as shown in Fig. 6, A and B, the images with higher power (10 kV) are included (Fig. 6, C and D, arrow). At this higher power, the difference in fiber cell width between CRYAA_{WT} and CRYAA_{N101D} was also observed. The widths among fiber cells were not uniform, and the average widths of straight fibers were increased in lenses of CRYAA_{N101D} mice compared with CRYAA_{WT} mice.

The SEM micrographs of 9-month-old lenses showed concentric radial fiber cell columns in WT lenses (Fig. 6E), which were unlike compaction of concentric layers of elongated fiber cells in the inner cortical region in CRYAA_{N101D} lenses (Fig. 6F). This again suggested substantial changes in the organization of lens cortical fiber cells in lenses with deamidated αA-crystallin. Together, the results suggested that relative to WT αA, the expression of αAN101D-transgene resulted in distortion of the tightly packed fiber cell arrays and its abnormal interactions with fiber cell membranes.

Crystallin Profiles of Lenses from CRYAA_{WT} and CRYAA_{N101D} Mice—Because lenses of CRYAA_{N101D} mice 5 months old and older showed significant defects and morphological changes in their fiber cells and their membranes compared with age-matched lenses of CRYAA_{WT} mice, both the relative distribution of αA-crystallin in WS and WI protein fractions and levels of crystallins (α1-, β-, and γ-crystallins) were determined. To determine the relative association of His-tagged WT αA/αAN101D-transgene in WS, WI, and membrane fractions, these fractions were isolated from lenses of 3-, 5-, and 7-month-old CRYAA_{WT} and CRYAA_{N101D} mice and analyzed by the Western blot method using anti-His and anti-AQP0 antibodies (Fig. 7). Relatively higher levels of His-tagged WT αA/αAN101D-transgene and their truncated species were detected in the lens membrane fractions of CRYAA_{N101D} mice of all of the above three ages compared with CRYAA_{WT} mice (Fig. 7, compare lanes 3 and 6 of A with lanes 3 and 6 of C). This suggested a greater association of His-tagged αAN101D-transgene with membrane compared with αA WT. Further, the Western blot analysis following immunoreactivity with anti-AQP0 antibody showed aggregation of AQP0 protein species of >43 kDa in the membrane fractions of lenses from the above three ages of CRYAA_{N101D} mice compared with lenses of age-matched CRYAA_{WT} mice (Fig. 7, compare lanes 3, 6, and 9 of B with lanes 3, 6, and 9 of D). Fractionation of WS protein from CRYAA_{N101D} and CRYAA_{WT} lenses by size exclusion HPLC using the multi-angle laser scattering (MALS) method (A280 absorbance) showed a negligible α-crystallin peak and reduced peaks of β- and γ-crystallins in the former compared with the latter (data not shown). MALS analysis exhibited 1.2 × 10^2 and 5.9 × 10^2 Da molecular mass of α-crystallin from CRYAA_{N101D} and CRYAA_{WT} mouse lenses, respectively. This could be due to aggregation of αAN101D-transgene compared with WT αA-crystallin. However, the computed molar mass of the deamidated α-crystallin peak had a ±32% error compared with WT α-crystallin peak with a ±4% error.

To determine the relative distribution of WS and WI proteins in lenses of CRYAA_{WT} versus CRYAA_{N101D} mice, their amounts were determined. Both 5- and 7-month-old lenses showed a higher level of WS proteins relative to WI protein in lenses of CRYAA_{WT} mice, but the ratio between WS and WI proteins was reversed in the lenses of CRYAA_{N101D} mice (Fig. 8). This was consistent with the results from Western blot analysis of WS and WI proteins from these lenses using an anti-His tag antibody as described above (Fig. 7). Taken together, the results suggested that the deamidation of αA-crystallin plays a role in altering protein conformation, which results in their aggregation and water insolubilization.

DISCUSSION

PTMs, such as glycation, phosphorylation, deamidation, and oxidation, can lead to aggregation and cross-linking (8–11, 34), but at the cellular level, these could be implicated in changes of lens fiber cell folds and breaks (35), extracellular spaces (36), suture subbranches (33), and fiber cell compaction (37, 38). These cellular changes could be potential sources for light scattering, leading to age-related cataract development. For example, the fiber cell compaction is believed to play a significant role in light scattering in diabetic versus non-diabetic lenses (38). Although voluminous literature has suggested that the deamidation of Asn and Gln in crystallins causes alterations in their hydrophobicity, tertiary structures, interaction with other proteins, aggregation, and insolubilization (10, 21, 22, 39–42), so far no clear molecular mechanism has emerged that could implicate the deamidation-induced changes in crystallins in vivo leading to cataract development. Therefore, our present study, which introduced a charge due to deamidation during development in αA-crystallin transgene in transgenic mice to determine lens phenotypic and protein solubility changes, is significantly the first unique study. This model directly links deamidation to cortical cataract in an in vivo model. The major findings of the study were that, relative to WT αA-crystallin, the expression of αAN101D-transgene reduced the lens size and crystallin solubility, altered αA interaction with the membrane, caused persistence of nuclei in the inner cortical region and degeneration of fiber cells, and led to mild cortical opacity. Further, the prevalence of in vivo deamidation of αA Asn-101 to Asp in 5-month-old CRYAA_{WT} and CRYAA_{N101D} mice was investigated by two-dimensional gel electrophoresis followed
by identification of spots by the Q-TRAP mass spectrometric method. A clear shift in the charge of \(/H9251A\) was observed in protein extracts from CRYAAN101D mouse lenses compared with CRYAAWT mouse lens extract. The deamidated species among all of the \(/H9251A\) spots was searched using MASCOT (Matrix Science). The deamidated species was quantified by using the ImageJ (1.42q) program (National Institutes of Health; available on the World Wide Web). The analysis showed that \(/H9251AN101D\) constituted 14 and 14.2% of total \(/H9251A\) in the WS and WI proteins, respectively, in the lenses of CRYAAN101D mice.

\(/H9251A\)-crystallin cDNA tagged with a His7 tag epitope at its N terminus (obtained from Dr. Mark Petrash) (33) allowed us to immunologically distinguish the transgene product from the endogenous mouse \(\alpha\)A-crystallin. Additionally, a previous study by Petrash and co-workers (33) showed that the incorporation of His7 tag at the N-terminal end did not measurably alter the functional properties of \(\alpha\)A-crystallin. Our past biochemical studies carried out in vitro with deamidated \(\alpha\)A/\(\beta\) showed mixed results related to their solubility. It is therefore a possibility that the His tag could affect the solubility property of His-tagged \(\alpha\)A-crystallin in our study. However, because both WT and the deamidated \(\alpha\)A were His-tagged, the His tag effect on the \(\alpha\)A solubility was assumed to be normalized for all practical reasons.

The reduced size of the lenses in CRYAAN101D mice was consistent with previous reports suggesting a potential role of
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FIGURE 8. Comparative distribution of WS and WI protein in lenses of CRYAA<sub>WT</sub> and CRYAA<sub>N101D</sub> mice. Compared with CRYAA<sub>WT</sub> mice, the lenses of CRYAA<sub>N101D</sub> mice showed relatively lower levels of WS proteins but higher levels of WI proteins. Also, higher levels of aggregated αA-crystallin in the WS/WI fraction of CRYAA<sub>N101D</sub> lenses were observed compared with lenses of CRYAA<sub>WT</sub> mice. The levels of WS/WI proteins were confirmed by using anti-His monoclonal antibody, as shown in Fig. 7, suggesting that the expression of a deamidated αA-crystallin causes protein insolubility. Error bars, S.D.

αA-crystallin in lens growth (43–46). In αA-crystallin knock-out mice (44) and in both αA and αB double knock-out mice (45), a relatively smaller lens than in WT mice was attributed to a decrease in the net production of epithelial cells. These studies also speculated that in vivo expression of αA in the lens epithelium protected these cells against cell death during mitosis. This was supported by a report showing that an addition of αA-crystallin to lens epithelial cell cultures induced cell differentiation (46). Because only heterozygous mice were examined in our study, it is possible that mice with a double copy of the deamidated transgene would exhibit significantly smaller lens size. A transgenic mouse model expressing the αAR116C mutant, which is associated with autosomal dominant cataract (33), did not show any of the above morphological defects; however, it exhibited posterior cortical cataracts and abnormalities associated with posterior suture. This study, along with our present study, suggests that the presence of a mutation or PTM in the conserved α-domain of αA-crystallin contributes significantly to lens opacity.

Bright field microscopic analysis showed posterior suture defects in lenses of 1–7-month-old CRYAA<sub>N101D</sub> mice compared with CRYAA<sub>WT</sub> mice. The lens suture lines are seamlike structures formed when tips of elongated fiber cells overlap with tips from opposite fiber cells (47). Because the suture line patterns represent precise migratory elongation and destination of fiber cells in the lens, any defects in elongation or migration of fiber cells are represented by irregular or excessive suture branches. Also, the suture defects are believed to affect lens optical quality (48, 49) and might lead to cataract development (33, 50). The abnormal suture lines in the lenses of 1-month-old CRYAA<sub>N101D</sub> mice, compared with CRYAA<sub>WT</sub> mice, represented an early differentiation defect in fiber cells.

A novel OCT technique that provides non-invasive high resolution, direct, and real-time visualization of tissues has previously been used to quantify the lens cataract density (51). OCT analysis of lenses of CRYAA<sub>N101D</sub> mice showed relatively more opacity in the superior cortical region at 7 months of age compared with clear lenses of age-matched CRYAA<sub>WT</sub> mice. The lenses from 1–3-month-old CRYAA<sub>N101D</sub> mice showed posterior suture defects; however, no opacity during the OCT examination was observed. This suggested that the lens opacity was a delayed process but a consequence of specific effects of the expression of deamidated αA transgene protein. The results are supported by the fact that the cortical spokes could develop within the cortex of the lens (52) but usually do not cause visual symptoms unless opacity develops around the visual axis. Several similar transgenic expressions of different lens proteins with fiber cell abnormalities have been reported without them leading to lens opacity (53, 54). For example, an intermediate filament protein knock-out mouse showed perinuclear haze and decreased lens optical quality but no lens opacity (55). Taken together, clearly the age-related changes in the lens could lead to a loss of optical quality but not always cause cataract.

The suture defects were observed at an early age (i.e., 1 month of age) in our study, which suggested abnormal fiber cell differentiation in lenses containing deamidated αA. However, the immunohistochemical and SEM analyses showed such lens abnormalities only at later ages of mice (i.e., 5 months or older). The fiber cell differentiation abnormalities, including irregular fiber cell shapes, disordered packing, and abnormal extracellular spaces, were significantly pronounced at 7 months of age in lenses of CRYAA<sub>N101D</sub> mice compared with CRYAA<sub>WT</sub> mice. The lenses of αA- and αB-crystallin double knock-out mice also exhibited similar fiber cell morphological abnormalities up to more than 13 months of age (45). On the contrary, a study with CRYAA<sub>R116C</sub> transgenic mice showed cortical cataracts and posterior subcapsular defects but did not show any age-related changes in cataract progression (33). Thus, the potential mechanistic roles of loss of either αA- or αB-crystallins in fiber cell defects in the above knock-out mice or in our study remain to be determined.

Differentiation of epithelial cells to fiber cells is a well-programmed developmental process with major morphological changes and extensive changes in gene expression. The differentiation is accompanied by the expression of fiber cell-specific markers, such as β- and γ-crystallins, as well as the intermediate filament proteins CP49 and filensin (56). In light of this, decreased levels of crystallins (α, β, and γ) observed in our study are significant and imply that the presence of αAN101D-transgene in vivo induced changes in crystallins and other lens proteins leading to cataract development. During epithelial terminal differentiation, several characteristic processes occur, which include the withdrawal of epithelial cells from the cell cycle, extensive elongation of fiber cells, development of specialized cell junctions, loss of nuclei and intracellular organelles, and accumulation of crystallins, particularly αA-crystallin, which constitutes ~20% of newborn human lens protein. Our results did not provide evidence for the above mentioned processes; however, attenuation of denucleation even in 1-month-old CRYAA<sub>N101D</sub> mice suggests that the presence of deamidated αA affects fiber cell differentiation. Among several growth factors that show proliferation of epithelial cells, only fibroblast growth factor has been shown to stimulate differentiation of epithelial cells to fiber cells by stimulating cell elongation and expression of fiber cell-specific proteins (57). However, members of the transforming growth factor-β (TGF-β)
superfamily and Wnt/β-catenin-signaling pathway have also been implicated in terminal differentiation and fiber cell elongation (58, 59). Any improper execution of the programmed process of differentiation of epithelial cells into fiber cells can lead to abnormalities in fiber cells and also to cataract development, as reported previously (60, 61). Determination of whether pathways controlled by the above described growth factors are affected by the expression of deamidated αA-crystallin requires further investigation.

Presently, the lack of experimental evidence led us to speculate about reasons for abnormal fiber cell morphology as seen in lenses of CRYAA<sub>N101D</sub> mice compared with CRYAA<sub>WT</sub> mice. The deamidation of Asn at residue 101 in αA, which is located in the β-strands of the conserved α-crystallin domain, not only imparts an extra negative charge to the protein but also adds an extra carbon to the polypeptide backbone. This change in αA-structure can be deleterious and may explain some of the morphological changes at the bow region, where αA is predominantly synthesized in epithelial cells. The deamidated αA-crystallin per se with an altered structure could form abnormal aggregates with other crystallins, have altered interactions with αB and/or other crystallins, and accumulate with damaged proteins due to its reduced chaperone activity, as reported previously in our in vitro studies (21).

Relative to confocal analysis, the SEM analysis showed more revealing changes in the organization of fiber cells in lenses from CRYAA<sub>N101D</sub> mice compared with CRYAA<sub>WT</sub> mice. These changes included altered cortical fiber cell membranes, loss of interdigitations that usually arrayed along the fiber cell length, and a greater width among fiber cells in 5-month-old and older mice. The results suggested that the deamidated αA might also cause partial swelling of membranes due to osmotic imbalance initiated by an additional negative charge in the deamidated αA-crystallin. If circulation of fluids is perturbed, which is essential for homeostasis of the lens central fiber cells, it might eventually lead to lens opacity. Because the lens circulatory system is generated primarily by Na<sup>+</sup> from extracellular clefts between fiber cells, the introduction of additional negative charge by deamidated αA would disrupt this intracellular current and hence can contribute to the lens opacity.

Interaction between αA-crystallin and fiber cell membranes has been shown in previous studies (27, 28), and any alteration between the two must be due to altered structure of binding proteins and might explain abnormal structural changes in cortical fiber cells of lenses of CRYAA<sub>N101D</sub> mice compared with CRYAA<sub>WT</sub> mice. An in vitro study with the αA-R116C mutant showed a ~10-fold enhanced membrane binding property compared with WT αA (28). Although no such data exist for the αAN101D mutant, based on our present results, it is intriguing to consider that deamidation alters interactions between αA-crystallin and membrane proteins. Interaction between αA-crystallin and AQPO has been shown to be mostly hydrophobic in nature (27), and our previous in vitro studies have shown altered surface hydrophobic property of αA-N101D (21, 24) compared with the WT αA. Therefore, the increased association of αAN101D crystallin with AQPO might be a consequent effect. This greater association of αAN101D with membrane was consistent with the Western blot analysis, which showed greater presence of deamidated αA in membrane fractions of 3-, 5-, and 7-month-old CRYAA<sub>N101D</sub> mice compared with WT αA in age-matched CRYAA<sub>WT</sub> lenses. This was also consistent with aggregation of deamidated αAN101D compared with WT αA-crystallin because of the higher levels of the former in WI proteins of CRYAA<sub>N101D</sub> mice compared with CRYAA<sub>WT</sub> mice.

A higher level of WI protein and increased aggregation of deamidated αA in both WS and WI protein fractions was accompanied by relatively lower levels of α-, β-, and γ-crystallins in lenses of 5- and 7-month-old CRYAA<sub>N101D</sub> mice compared with CRYAA<sub>WT</sub> mice. However, whether the lower levels of crystallins were the result of transcriptional and/or translational control or due to their aggregation is presently not clear.

The present study showed that most notable changes in lens fiber cell morphology, membrane abnormality, and altered association of αAN101D-transgene with membranes began at 5 months of age in CRYAA<sub>N101D</sub> mice compared with CRYAA<sub>WT</sub> mice. Such changes did not occur or minimally occurred in lenses of 1-month-old CRYAA<sub>N101D</sub> mice except for an abnormality in the suture lines. Therefore, the results suggest that the deamidation affected both fiber cell differentiation and association of αAN101D-transgene with membrane with aging. However, because no investigation of comparative developmental changes in lenses of CRYAA<sub>N101D</sub> and CRYAA<sub>WT</sub> mice was carried out, whether lenses were developmentally affected due to αAN101D is presently unclear and is under investigation.

In summary, this study demonstrates for the first time that an introduction of a charge during development (deamidation of the Asn-101 residue to Asp) in αA-crystallin causes protein insolubility and loss of integrity of fiber cells and leads to a mild cortical opacity. At present, how the expression of deamidated αA-crystallin in the lens epithelium influences epithelial transitional zone cells (cells that are in transition to become differentiated fiber cells) remains unclear. In this study, fiber cells with deamidated αA showed attenuated denucleation, suggesting that the presence of deamidated αA influenced this process. This study also provides an opportunity to investigate the biochemical/molecular mechanism of how deamidation of Asn-101 to Asp in αA-crystallin contributes to cortical cataract development.

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