Molecular Processes Implicated in Human Age-Related Nuclear Cataract

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Human age-related nuclear cataract is commonly characterized by four biochemical features that involve modifications to the structural proteins that constitute the bulk of the lens: coloration, oxidation, insolubility, and covalent cross-linking. Each of these is progressive and increases as the cataract worsens. Significant progress has been made in understanding the origin of the factors that underpin the loss of lens transparency. Of these four hallmarks of cataract, it is protein-protein cross-linking that has been the most intransigent, and it is only recently, with the advent of proteomic methodology, that mechanisms are being elucidated. A diverse range of cross-linking processes involving several amino acids have been uncovered. Although other hypotheses for the etiology of cataract have been advanced, it is likely that spontaneous decomposition of the structural proteins of the lens, which do not turn over, is responsible for the age-related changes to the properties of the lens and, ultimately, for cataract. Cataract may represent the first and best characterized of a number of human age-related diseases where spontaneous protein modification leads to ongoing deterioration and, ultimately, a loss of tissue function.

Keywords: protein-protein cross-linking, oxidation, temperature, amino acid instability, eye

Age-Related Nuclear Cataract (ARNC)

This review will focus on the molecular aspects of human cataract formation and will allude to other aspects, such as epidemiology, only when it is relevant to the main topic. A recent summary of epidemiologic literature can be found elsewhere.[1–3] Since other reviews on age-related cataract have been published (see, for example, Refs. 4 and 5), this manuscript will not aim to be a comprehensive summary of all the literature but will instead primarily deal with the most recent data, in particular biochemical advances that have led to a greater understanding of this condition.

As the name indicates, aging is a precondition for the development of ARNC. ARNC is the most common of the three types of cataract, the other two being cortical and posterior subcapsular. The vast majority of people who succumb to ARNC are older than 60 years of age, and the incidence increases with age thereafter.[6] Therefore, it is not possible to discuss the molecular reactions responsible for ARNC without also characterizing the events that take place in the normal human lens with age. A picture has emerged that ARNC becomes apparent once a certain level of age-related change within the lens has accumulated. Past this point, ARNC may be inevitable.

Particular attention will be directed toward outlining the mechanisms responsible for protein-protein cross-linking in human lenses. It is in this arena that much recent progress has been made, and it has become apparent that there is a variety of amino acids and chemistries involved.

ARNC and Aging

Although aging is associated with marked changes to the properties of the human lens, in the vast majority of adults, the lens remains transparent past middle age.[7] This is not to imply that vision in a middle-aged person is as clear as that of a younger person. Nevertheless, despite image quality being lessened in a mature adult, light scattering of the lens is insufficient to necessitate its removal.

What then are the processes that underpin lens opacification? It should be stated that not all of these processes and the chronological sequences that transform a highly altered aged lens into one that is a cataract are precisely known. There are, however, a number of correlations that can be knitted together to develop a compelling story.

Loss of a Chaperone

After the fourth decade, the amount of free α-crystallin in the center of the human lens declines to zero.[8–10] It appears that the allocation of α-crystallin that was provided to us at birth has been used in binding to other proteins that denatured and unfolded over time.[11] Some α-crystallin in adult lenses has been truncated,[12,13] but most appears to be bound to other proteins that have denatured, since many studies have shown that the amount of high molecular weight (HMW) protein aggregates increases as the amount of the free α-crystallin in the lens declines.[14] This observation is entirely consistent with α-crystallin acting as a molecular chaperone.[15]

Therefore, in our 40s the center of our lenses becomes incapable of sequestering the proteins that continue to degrade with age. Presbyopia becomes apparent at this time, and after a decade or more, the consequences of this lack of protection for clear vision appear to be dire.[16]

Binding of Crystallin Aggregates to Cell Membranes

An important ramification of an absence of free (unbound) α-crystallin is that protein aggregates bind to the fiber cell

membranes.[11,17,18] The means by which this linkage of events occurs is unclear, but the process can be readily followed using density gradient centrifugation.[11,18] At middle age, the banding pattern changes dramatically. After age 40, a large proportion of total lens crystallins is found in bands that also contain the lens membrane lipids. Between the ages of 40 and 60, approximately 45% of total protein is associated with these high-density membrane bands. Above age 60, this increases to approximately 57%.

The exact mechanism of binding is still not known. Presumably, it could involve noncovalent attachment to the remains of cytoskeletal components that coat the inside surface of the fiber cells or possibly to membrane lipids or integral membrane proteins. Some covalent binding may be involved. Interestingly, analysis of the bound aggregates revealed a large proportion of α-crystallin, with a relatively higher abundance of the phosphorylated forms.[11,18] Such bound aggregates may represent HMW complexes where α-crystallin is bound to other lens proteins that have unfolded.[19]

The Lens Barrier

There appear to be functional consequences linked to this massive binding of crystallins to membranes. The most important leads to impairment of cell-to-cell transport within the lens interior. This has been termed the "lens barrier"[20] and has been characterized in whole human lenses in vitro by tracking the movement of both glutathione (GSH) and water.[20,21] In each case, these small molecular weight compounds move freely in the lens cortex, but further inward diffusion is impeded at an anatomic region that corresponds in size to the lens at birth.

Thus, the human lens cortex becomes estranged from the lens nucleus in the fifth decade of life. Two things result once the barrier forms at middle age. Firstly, small molecular weight compounds cannot readily enter the quiescent interior from the metabolically active lens cortex. Of most importance is the primary lens antioxidant, GSH. GSH is synthesized in the lens cortex, and oxidized GSH is reduced here.[22] Such an impediment therefore renders the macromolecules in the lens core susceptible to oxidation.

Secondly, metabolites cannot exit the core into the lens cortex. At first sight, this diminished egress may seem to be of less importance than that of the altered redox state; however, longer dwell times of unstable compounds in the interior will result in an increased generation of reactive chemicals. A number can be listed and include the endogenous UV filter compounds that are synthesized within the lens from the amino acid tryptophan. Some, such as 3-hydroxykynurenine, are highly reactive and in the presence of oxygen, covalently modify proteins leading to their coloration.[23] Other modifying metabolites present in the lens are oxidized GSH and dehydroascorbate, both of which are known to covalently attach to proteins.[24–26]

It is probable that decreased ingress and a lower rate of metabolite egress from the nucleus act synergistically to promote a greater extent of covalent protein modification in the incipient cataract lens. One factor in particular will exacerbate covalent modification by reactive metabolites, and this is a lack of adequate GSH. GSH has two crucial functions; it impedes autoxidation of reactive small molecules and competes with nucleophilic groups on proteins for attachment to the metabolites.[27,28] These various processes are summarized diagrammatically in Figure 1.

Having enumerated the molecular processes that may be responsible for initiating ARNC, it is necessary to discuss the events that were responsible for protein decomposition in the human lens.

What Age-Related Changes to the Structural Proteins of the Lens Cause Them to Unfold and Bind to α-Crystallin?

Inexorable Decomposition of Lens Proteins Accompanies Aging

It is now well established that large-scale breakdown of lens proteins takes place as we age. Research over many years has enabled researchers to characterize and to quantify these modifications as a function of age. One striking finding has been that the modifications are spontaneous: they require no enzymes. In retrospect, this should not have been surprising, since the center of the lens appears to be either devoid of active enzymes,[29–31] or if present, retain only trace activity.

This region has been present before the time of birth with no turnover of macromolecules.[32] Therefore, it would be remarkable if active enzymes remain in the center of adult human lenses, given that even by age 20, all nuclear proteins will have been subject to thermal denaturation for over 175,000 hours! Despite the fact that this is unlikely, other enzymes and transporters should be examined for activity in human lenses to corroborate this hypothesis. In relation to this, the detection of macromolecules does not mean that they are active. For example, glucose-6-phosphate dehydrogenase can be detected in adult human lenses by Western blotting, but the molecules are denatured and inactive.[31]

The main processes that take place are racemization, isomerization, peptide bond cleavage, and cross-linking.[33] Deamidation of Asn, and to a lesser extent Gln residues, is intimately linked to racemization and isomerization. Aspects of these age-related modifications have been discussed elsewhere,[34–42] so will not be described in much detail here, with the exception of cross-linking; knowledge of the processes underlying cross-linking has increased substantially in the past several years.

Crystallin Decomposition: Proteins in the Adult Lens Are Not the Same as Those That Were Present at Birth

Two-dimensional gel electrophoresis clearly demonstrates that the proteins in the adult human lens are different from those of fetal lenses.[42,43] It turns out that lens proteins are subject to many age-related covalent and noncovalent changes.

Racemization/Isomerization

A remarkable feature of the adult human lens is that it remains transparent, despite a huge amount of crystallin degradation. One statistic serves to illustrate this point. By the age of 60 years, there are between two and three D-amino acids within *every* polypeptide chain in the lens (Fig. 2).[34] Of course, this is an average figure and masks the fact that some crystallins are more highly modified than others. Considering how straightforward the method of analysis, another surprising feature of this discovery is how long it took for this finding to become apparent. It is a relatively simple matter to separate D- and L-amino acids by HPLC after acid hydrolysis and therefore to quantify the two isomers. Asp and Asn are the amino acid residues most susceptible to racemization, with significant Ser also being present in adult lenses as the D-isomer. One aspect that remains unexplained is why the rate of racemization is fastest in childhood prior to teenage years (see Fig. 2).[34] Although papers were published in 1970s that showed the potential relevance of racemization for human cataract,[44–46] for

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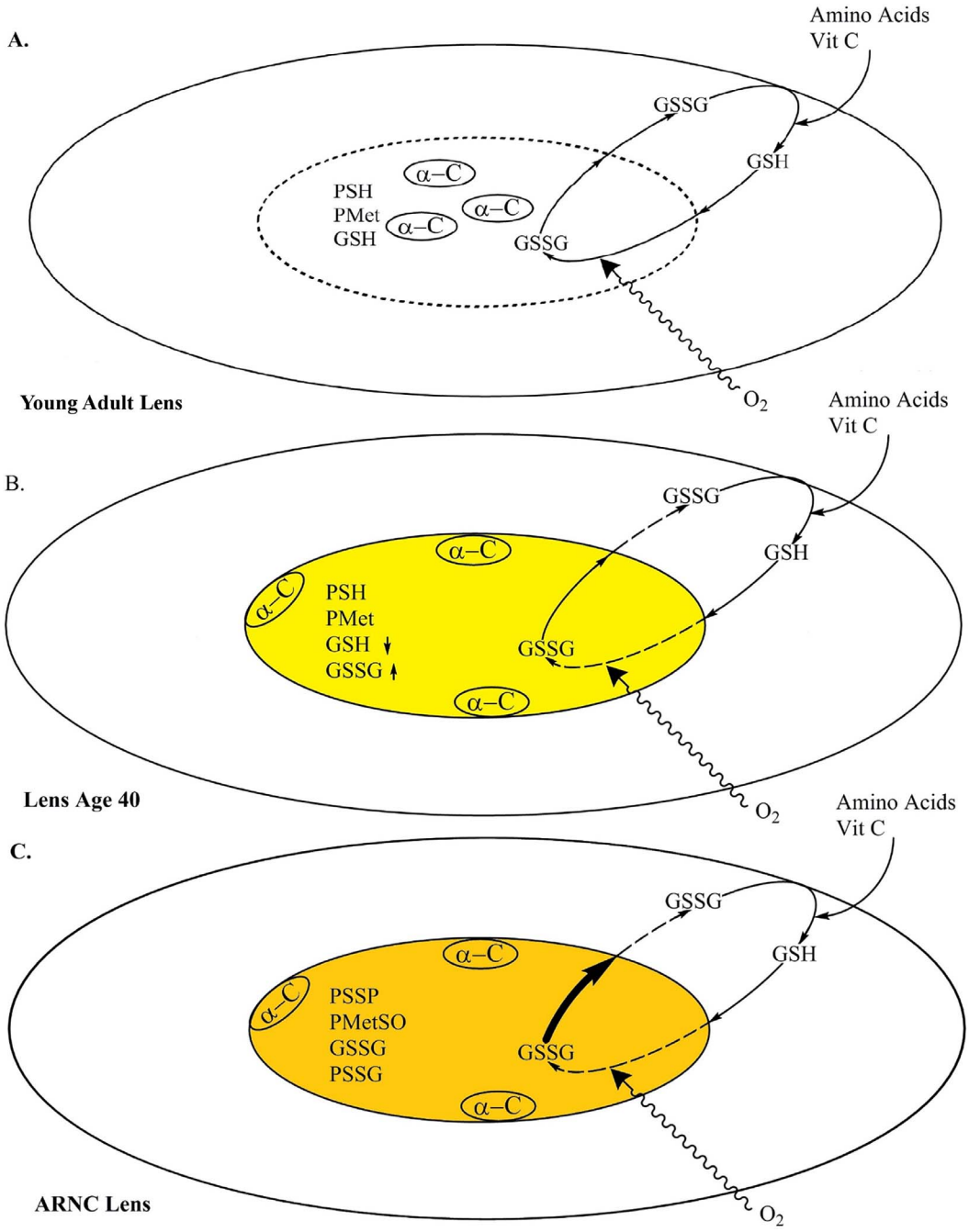

Figure 1. (**A**) A diagrammatic representation of the main processes underpinning ARNC formation and how this is linked to human aging. (**B**) At middle age, an internal barrier to diffusion forms at the anatomic zone that corresponds to the size of the lens at birth. From this age onward, GSH access to the nucleus becomes limiting. Oxygen is still able to access the nucleus, so the nuclear proteins become susceptible to oxidation. GSH levels fall. (**C**) Once ARNC commences, protein-protein disulfide levels increase dramatically as do levels of methionine sulfoxide, and mixed disulfides also can be detected. The lens barrier appears to be due to the large-scale binding of α-crystallin aggregates, formed by binding of α-crystallin to proteins in the lens as they denature (α–C). These attach to the inner surfaces of the fiber cell membranes, thus occluding the membrane pores. Proteins become colored, particularly in the lens center, as UV filters and other reactive metabolites (e.g., ascorbate) become bound and oxidize.

many years the study of amino acid racemization in the lens was dominated by Fujii's group in Japan.[37,38,47] It was some time before other lens researchers came to appreciate its importance.

When ARNC lenses were examined using the HPLC methodology, it became clear that the extent of protein racemization was greater in these opaque lenses (Fig. 2).[34] The conversion of L-Asp/L-Asn to the D-Asp isomers was ~20% to 40% more than that detected in normal lenses. The levels of D-Ser were also significantly higher. One conclusion of this study was that racemization may be a key process that underpins the development of ARNC. Put simply, once protein racemization reaches above a certain level, the lens may become opaque. A related possibility is that opacification may result if the racemization levels are raised significantly by comparison to those present in normal age-matched lenses. While such

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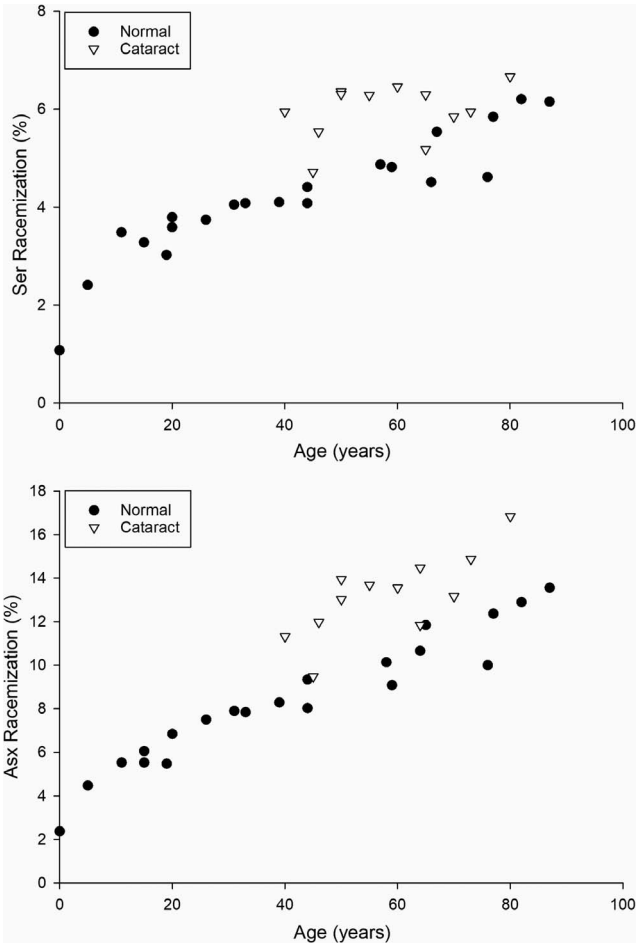

Figure 2. Racemization of Asp/Asn and Ser as a function of lens age. Over time, L-Asp and L-Asn residues in lens proteins convert to D-Asp or D-isoAsp. The degree of racemization is huge, corresponding to 1 to 2 Asp/Asn residues in every lens protein by age 60. This would be expected to lead to large-scale protein denaturation. D-Ser levels in lens proteins accumulate following a similar pattern with age, although the absolute levels are lower than those for Asp/Asn. For comparable age-matched cataract lenses, the racemization levels for both Asp/Asn and Ser are significantly higher, suggesting that spontaneous racemization plays a key role in ARNC formation. Interestingly, the levels of D-Ser are higher at age 40 to 60 years in cataract lenses, but in the older cataract lenses they do not appear to be much increased above those of age-matched normal lenses. The time zero value represents artifactual racemization due to the process of acid hydrolysis. These graphs were reproduced from data published in Hooi and Truscott.[34]

postulates are appealing, there are likely to be other complicating factors, and these will be enumerated below.

Deamidation

By virtue of the fact that the process of deamidation involves the formation of a cyclic succinimide intermediate that can more readily racemize, deamidation of Asn residues is also associated with significant racemization.[48]

When proteomic methods were employed to examine the crystallins from normal and age-matched ARNC lenses, it was found that most Asn residues in the individual proteins underwent deamidation to similar extents in the two lens groups.[35] In addition, the extent of deamidation of Asn was three times greater than that of Gln (Asn, 22.6% ± 3.6%; Gln, 6.6% ± 1.3%).[41] There were, however, some notable exceptions.

Can Deamidation of Certain Key Asn Residues Precipitate Cataract?

Detailed proteomic analyses showed that some Asn residues were consistently more highly deamidated in ARNC lenses than in age-matched controls. This was true across the age range from 40 to older than 80 years of age. Asn 76 in γS- crystallin was one such site.[35]

It is not easy to assess whether such site-specific modification is actually a significant determinant of human cataractogenesis. This scenario is certainly not implausible since there are hundreds of instances of congenital cataract resulting from one amino acid substitution in a single crystallin.[49] It could be that an amount of deamidation above a certain level at these specific sites within an already highly modified crystallin background acts as "the straw that broke the camel's back" to cause these lenses to opacify.

Asp 58 in αA-crystallin is another site where the extent of racemization is significantly greater in cataract lenses than in aged-matched clear lenses.[50]

A significant factor responsible for protein denaturation in older lenses is that the altered Asn (and Asp) residues have become mostly D-isoAsp residues.[35,51,52] This unusual amino acid is not only racemized at the alpha carbon, but also an extra methylene group has effectively been inserted into the polypeptide backbone.

In model studies, deamidation of β-crystallins disrupted interactions with other crystallins, and α-crystallin was unable to prevent some deamidated crystallins from becoming insoluble.[39] Similar studies revealed comparable structural consequences for the closely related γ-crystallins.[53]

Isomerization of Ser and Asp and Its Effect on Protein Structure

The effect of isomerization of specific Asp and Ser residues in α-crystallin has been studied by Julian's group.[52] Epimerization of Ser 162 in αA-crystallin was found to weaken intersubunit interactions, as did isomerization of Asp 62 in αB-crystallin. Isomerization of Asp 109 in αB-crystallin disrupted a salt bridge with Arg 120, a link that, when impaired, is known to influence oligomerization in other disease states. This demonstrates that isomerization at very few sites, such as those detected in aged and cataract human lenses, can have profound effects on structure and function of proteins such as this key lens chaperone.

Peptide Bond Cleavage

Scission of peptide bonds is widespread in adult human lenses and results in truncated crystallins, as well as a multitude of smaller peptides.[13,54] One of the pioneers in this field was Srivastava,[55] who found that HMW proteins from human lenses contained truncated polypeptides and that age-related polymerization could lead to their insolubilization.

In line with this observation, some peptides have been shown to affect protein aggregation[56] and, if present in sufficiently large amounts, could accelerate lens opacification. Other peptides can lodge in fiber cell membranes and may impair transport processes in older lenses.[57]

The majority of cleavages are due to spontaneous processes driven by the chemistry of amino acid side chains.[58] This is not to imply that peptidases are not present in the lens, since like in other tissues, proteolytic activity is required for normal cellular metabolism. For detailed accounts of lens proteolytic enzymes see Santhoshkumar et al.[59] and Sharma and Santhoshkumar.[60] It is unlikely that active proteases exist in the center of adult human lenses because, as elaborated above, in the known absence of renewal, this would mean that enzymes

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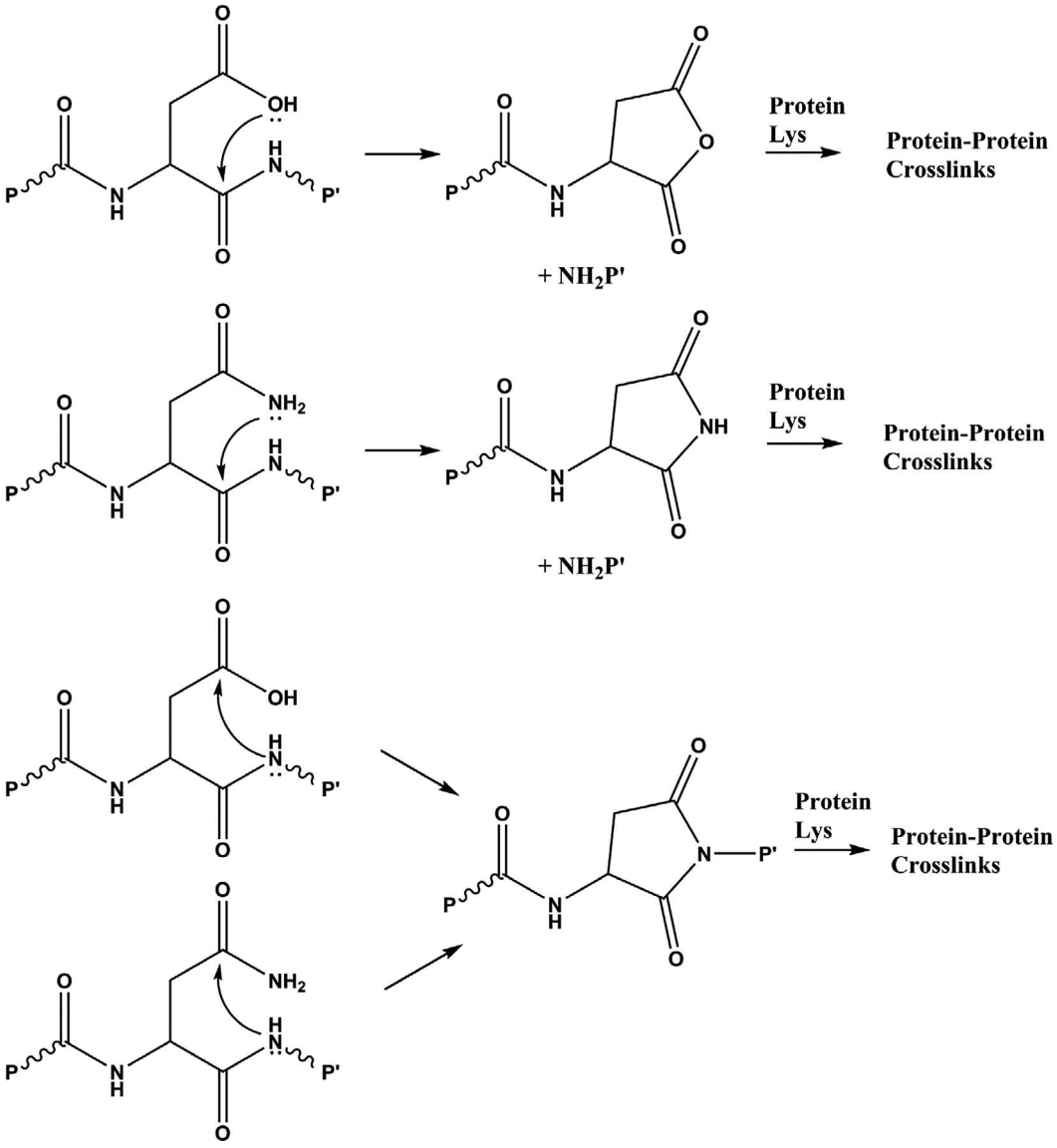

Figure 3. Lens proteins cross-link due to the spontaneous decomposition of some amino acids: aspartic acid and asparagine. The side chains of Asp and Asn can attack the peptide bond on the C-terminal side causing peptide bond cleavage and generating a C-terminal succinimide (Asn) or a C-terminal anhydride (Asp) as shown. These cyclic intermediates can react with Lys residues to produce covalent cross-links. In addition, over time, some Asn and some Asp residues cyclize by attack of the peptide bond nitrogen atom. The internal succinimides thus formed are also susceptible to attack by Lys residues forming covalent cross-links. Since succinimides are also intermediates in isomerization and racemization, this scheme links cross-linking with isomerization/racemization.

would have to survive thermal denaturation at 35°C for tens of thousands of hours.

Two amino acids, Asn and Ser, appear to be responsible for most peptide bond cleavage of crystallins. Spontaneous cleavage adjacent to Asp is also observed (see Fig. 3). Attack of the Asn amide side chain on the C-terminal peptide bond results in hydrolysis. This has been demonstrated clearly for the lens membrane protein aquaporin 0.[61]

Peptide bonds adjacent to Ser residues are also common sites for spontaneous cleavage. In this case, the bond on the N-terminal side of Ser is split. Once the key catalytic role played by zinc was discovered,[62] modeling of this process using peptides could be investigated.

In the lens, peptide bond scission next to Ser and Asn is followed by "laddering," that is, further progressive peptide bond cleavage from both the C- and N-terminal ends of the newly formed truncated peptides. The chemistry responsible for some of these processes is still poorly understood. One reaction that takes place at the N-terminus of peptides is diketopiperazine formation, which entails the spontaneous and sequential loss of dipeptides.[63] Detailed analysis of αB-crystallin from cataract lenses showed clear evidence of stepwise loss of dipeptides from a newly created N-terminus[64] that would be expected if such chemistry occurred in the lens. In the case of sequential loss of one amino acid from the N-terminal end, hydrolysis assisted by the free α-amino group is one mechanism involved,[62] but more comprehensive mechanistic studies are required.

Color: Unique to Human Cataract Lenses

A characteristic feature of human ARNC lenses is that they vary from deep yellow, to orange, to brown. Some advanced ARNC

lenses are almost black. Indeed, this clearly visible feature provided the basis for the Pirie/van Heyningen system of cataract lens classification.[65] This method proved to be remarkably useful and robust in the sense that a number of biochemical correlates were found to increase as the degree of cataract worsened, as judged by the increase in cataract from type I to type IV.[66–68] Some other animals also have colored lenses. For example, the lenses of squirrels[69] and some deep-sea fish are yellow[70,71]; however, in no case does any animal cataract model display the intensity or variety of colors associated with advanced human nuclear cataract.

Color and UV Filters

The observation that cataract lens coloration is unique to primates prompted some investigators to search for other human-specific lens attributes that could explain this. It was discovered that the primate-specific UV filter pathway was responsible.[5] van Heyningen identified 3-hydroxykynurenine glucoside[72] as the major human lens UV filter compound.

Truscott's[73] group investigated the UV filter pathway in human lenses over a period of years. To summarize, it was demonstrated that the major UV filter compound, 3-hydroxykynurenine glucoside, was made in the lens from the essential amino acid tryptophan and that intermediates in the pathway, kynurenine and 3-hydroxykynurenine, were also present.[73] Each of these intermediates was found to be unstable. At neutral pH, they undergo spontaneous deamination to yield the corresponding αβ-unsaturated ketones. Such ketones are susceptible to nucleophilic attack, typically by the amino groups of proteins.[74]

As a consequence, the amount of kynurenine and 3-hydroxykynurenine bound to proteins in the intact lens increases with age.[75] This provides an explanation for the age-dependent yellowing of the human lens, which, due to selective filtration of light, is associated with an altered perception of color.

Although these findings explain normal lens coloration and why it increases with age, could this process also be involved in the coloration of ARNC lenses? Although it is very difficult to prove, it seems probable that oxidation of the bound UV filters plays a pivotal role. This is particularly true for 3-hydroxykynurenine. This *o*-aminophenol is very prone to oxidation, and this can be easily demonstrated by dissolving a small amount in buffer at neutral pH. Over time in air, the solution becomes yellow, then increasingly brown. If protein is present during the autoxidation, the protein also becomes colored.[76]

3-Hydroxykynurenine-induced coloration is accompanied by polymerization, and the overall process is analogous to that responsible for tanning of cocoons in some species of moths, where the reactive chemical is a closely related *o*-aminophenol: 3-hydroxyanthranilic acid.[77]

Oxidation of *o*-aminophenols produces highly reactive *o*-quinoneimines,[78–80] which are subject to attack by nucleophiles. In the post-middle-aged human lens, where 3-hydroxykynurenine is attached to proteins by its side chain, oxidation of the aminophenol part will result not only in protein coloration, but also will lead to protein-protein cross-linking. If this process were significant, one would predict that covalent cross-linking and coloration should occur in parallel as ARNC proceeds. This indeed was found as the proportion of cross-linked polypeptides increased steadily as ARNC cataract advanced,[81] as monitored by the Pirie classification system, which is based on nuclear color.[65]

Color and Glycoxidation

Coloration and cross-linking of polypeptides in the human lens are thus closely linked, however the UV filter-based mechanism is not the only spontaneous process responsible for modifying crystallins in the lens. Reactive breakdown products of sugars and ascorbate have also been characterized in human lens proteins,[25,82,83] and some monosaccharide metabolites can cross-link proteins. As noted elsewhere,[33] it is peculiar that the levels of characteristic glycoxidation markers (e.g., carboxymethyl Lys) are very low in adult human lenses.[84] The meaning of this is unclear. Does this indicate that glycoxidation of lens proteins is a minor process, despite high (millimolar) glucose and ascorbate being in contact with the proteins for years, or is it that pathways of reaction in the lens are different from those in other tissues? The identification of a novel Lys cross-link denoted as K2P in cataract lenses possibly supports this latter hypothesis.[85]

Cross-linking

Aside from disulfide bond formation, the molecular mechanisms responsible for cross-linking of proteins in the human lens have become known only in the past few years.

As noted earlier, one of the mechanisms that operates in ARNC lenses involves covalent cross-linking by UV filters. As discussed elsewhere, Maillard-based cross-links have also been detected. Since a requirement for this to occur is an oxidative environment, this process does not appear to be significant in normal lenses. If oxidation takes place in normal lenses, it is minor, probably because GSH levels remain relatively high. The same reasoning applies to disulfide bonds. A detailed analysis revealed that while the level of disulfide bond formation in aged normal lenses was small, most cysteine residues in the crystallins were oxidized to disulfides in ARNC lenses. Only two buried Cys thiols remained in the reduced form in advanced cataract lenses.[86] It is probable that both inter- and intramolecular disulfide bonds are present in the proteins from ARNC lenses.[87]

Perhaps surprisingly, it has been found that a number of amino acids can participate in covalent cross-linking. In normal aged lenses, the spontaneous breakdown of phosphoserine, phosphothreonine, cysteine, asparagine, and aspartic acid to yield reactive intermediates that subsequently participate in cross-linking has recently been documented (Fig. 4). Research in this area is still ongoing.

Cross-linking via Phosphoserine, Phosphothreonine, and Cysteine

Spontaneous β elimination of phosphoserine produces dehydroalanine (DHA), which was predicted by Linetsky et al.[88] and is prevalent in the aged lens.[89] This electrophile is susceptible to attack by nucleophiles such as the thiol group of Cys and the ε-amino group of Lys (Fig. 4). Numerous sites of cross-linking through this mechanism have been elucidated.[88–91] The analogous hydroxylated amino acid, phosphothreonine, also undergoes the elimination of water, and attack on the corresponding double bond also leads to covalent cross-linking.[89,90]

DHA can also be generated in the lens from Cys residues, following oxidation to cystine, and neighboring amino acids can catalyze this reaction, rendering some Cys residues particularly susceptible to cross-linking.[91]

Cross-linking via Asparagine and Aspartic Acid

The precise details of this process are still being elucidated, however some aspects have been published.[92] Cross-linking can take place via the same cyclic intermediates that are known to act as intermediates in deamidation/racemization

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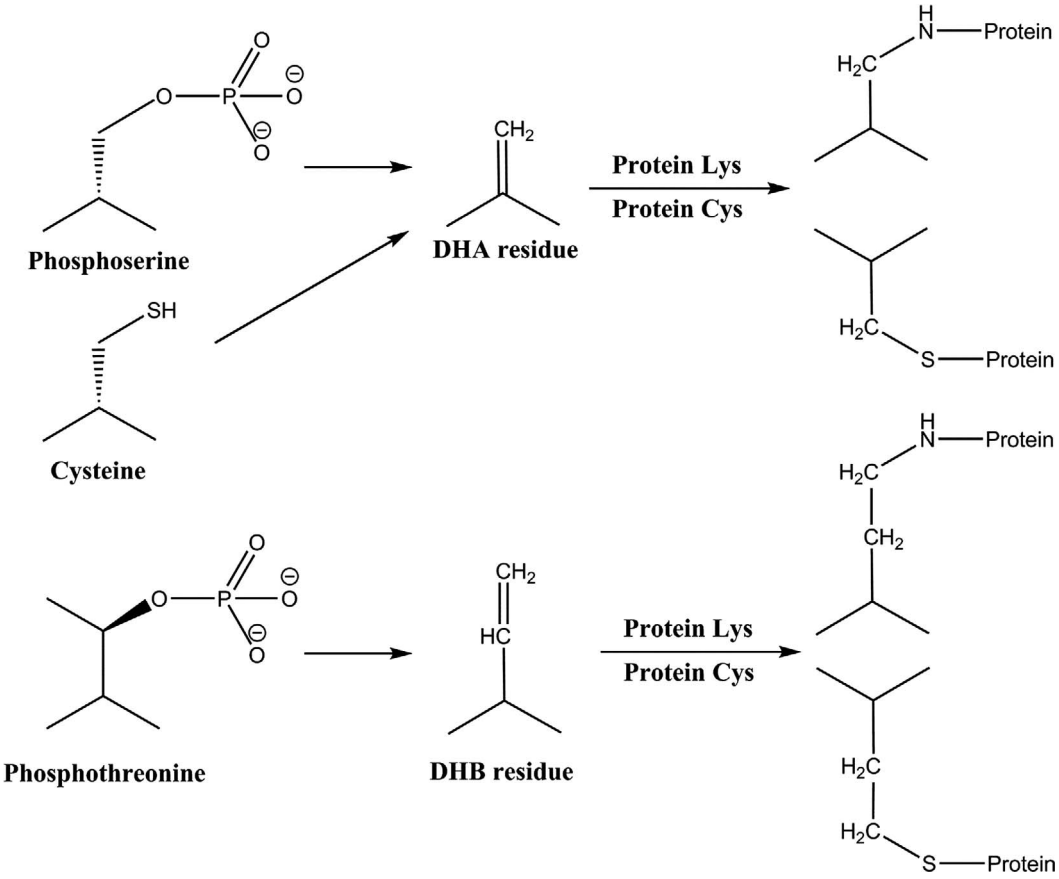

Figure 4. Lens proteins cross-link due to the spontaneous decomposition of some amino acids: phosphoserine, cysteine, and phosphothreonine. Over time, reactive dehydroalanine (DHA) residues are formed by β-elimination of phosphoserine and cystine residues. Other protein Cys or Lys residues can then attack the DHA residues forming covalent cross-links. Phosphothreonine residues decompose in an analogous manner, forming DHB, which undergoes analogous addition reactions. DHB, dehydrobutyrine.

and peptide bond cleavage (Fig. 3). Therefore, cross-linking mediated via Asn and Asp residues is a separate outcome of the same spontaneous chemistries that are responsible for adjacent peptide bond cleavage on the C-terminal side of Asp and Asn, as well as those involved in succinimide intermediate formation.

Insoluble Protein

Normal Aged Lenses

Aging of humans is linked with an increase in the amount of lens insoluble protein.[60] This is markedly accentuated with the onset of ARNC.

Insoluble protein can most readily be assessed by homogenization of lenses in buffer. To some degree the proportion of protein that is insoluble depends on the conditions used; however, this does not affect greatly the overall picture. Researchers are in agreement that insoluble protein increases with age,[93] even if it is not possible to relate the measurements from extraction experiments directly with the state of proteins in a highly concentrated cellular milieu.

Given the very high percentage of posttranslational modifications (PTMs) present in the crystallins and other proteins from aged lenses, it is perhaps not surprising that the physical characteristics of proteins, such as solubility, become altered. As described earlier, α-crystallin plays an important role as a chaperone in binding to the lens proteins as they denature, forming aggregates. It is thought that once aggregates become sufficiently large, they are more likely to be insoluble, although this is difficult to prove. In support of this hypothesis, detailed proteomic analysis of normal aged lenses found that the most abundant PTMs were deamidations and cysteine methylation and that the extent of deamidation was significantly increased in the water-insoluble fraction.[94]

ARNC Lenses

The development of ARNC is accompanied by an increase in a fraction of protein that is almost totally absent from normal age-matched lenses. This abundant fraction, which can account for 30% of total lens protein in type IV ARNC lenses,[68] is insoluble in 8 M urea but can be solubilized using 8 M urea containing dithiothreitol or mercaptoethanol. This feature suggested that interchain, and possibly to a lesser degree, intrachain, disulfide bonds were responsible for its formation. Significantly this urea-insoluble protein fraction was found to contain the majority of protein-bound pigment, and it was therefore designated as the "yellow protein fraction."[68] As well as being colored, it also is highly enriched in cross-linked polypeptides. Indeed, over 50% of the yellow protein fraction was composed of covalently cross-linked proteins. The characteristics of this cataract-specific fraction serve to illustrate the intimate linkage between coloration, cross-linking, and insolubility in ARNC.[66–68] This experimental finding is consistent with the long-standing hypothesis that accumulation of PTMs within crystallins is the cause of insolubilization. Experiments using recombinant β-crystallins

have shown that deamidation at critical sites can induce structural changes that disrupt their stability and lead to aggregation.[39] Deamidations at the surface also disrupt interactions with other crystallins.

Oxidation

Oxidation has been linked with the onset and progression of a host of diseases and conditions. In this regard, it is important to elucidate whether oxidation is involved in the onset of the condition or whether it is a late-stage event derived from breakdown in normal cellular metabolism. One must also be careful to eliminate experimental artifacts that can easily arise from handling and processing of tissues in the presence of air, and this is especially so in tissues in which the levels of antioxidants have been lowered.

Experiments showed that oxygen is present in the center of human lenses.[95,96] A steep oxygen gradient was maintained within the normal lenses, leading to PO_2 <2 mm Hg in the lens core. This element is highly diffusible and is soluble in organic solvents, suggesting that one route of diffusion into the lens center might be via the intricate network of fiber cell membranes. The vitreous is a major source of oxygen, and the concentration has been monitored before and after vitrectomy.[97] One reason given for rapid cataract formation following vitrectomy is that, post surgery, the lens is exposed to higher vitreal oxygen levels. In potential agreement with this hypothesis, leaving 3 to 4 mm of intact anterior vitreous behind the lens and not inducing posterior vitreous detachment during vitrectomy was associated with a fourfold lower risk of cataract surgery in the short term.[98] The impact of the vitreous and vitrectomy on lens properties and cataract was reviewed by Beebe et al.[99]

If it is possible to conceive of such an accolade, the "world record" for the most highly oxidized tissue in any animal is that of human advanced ARNC lenses. In type IV cataract lenses, greater than 90% of protein sulfhydryl groups are oxidized to disulfides, and approximately 40% to 50% of methionine residues are present as methionine sulfoxide.[66]

Loss of GSH in the lens interior was associated with this massive and progressive oxidation of proteins,[66] and this may be a prerequisite. In these advanced cataract lenses, despite the nuclear levels of GSH being low or undetectable, oftentimes GSH in the lens cortex was present at normal levels. This remarkable observation was the trigger for later research that demonstrated the presence of the lens barrier[20] after middle age.

Defenses Against Protein Deterioration

In humans, scores of enzymes protect DNA from degeneration and repair damage once it is detected.[100] In marked contrast, there are precious few defenses against protein breakdown. Protein isoaspartate methyltransferase (PIMT) appears to be our major safeguard. This enzyme detects sites of racemization of Asp and reverts D-Asp and L-isoAsp residues to L-Asp.[101] Unfortunately, it is not active against D-isoAsp, nor is it able to restore Asn residues since succinimide formation from Asn results in deamidation. Thus, a neutral Asn site on a protein is converted to a mixture of four negatively charged Asp isomers. Nonetheless, despite its limitations, PIMT appears to be a vital protector of long-lived proteins (LLPs), particularly those in the brain, because its deletion in mice leads to death after some weeks due to convulsions.[102]

Other enzymes, such as thioredoxin reductase, which requires NADPH as cofactor, can reverse oxidation of Cys residues, and methionine sulfoxide reductases restore oxidized Met residues. Clearly, these operate largely to reverse the outcome of oxidation, a feature that is not associated to a marked degree with normal lens aging.

The importance of preserving the thiol status of lens proteins is apparent from the fact that more than one damage repair system for thiols is present in lens cells. This includes the GSH-dependent glutaredoxin (thioltransferase) system for reducing protein-thiol mixed disulfides. Its deletion leads to increased sensitivity to oxidative stress,[103] as does knockout of glutaredoxin 2.[104] Knockout of a 15-kDa thioredoxin-like selenoprotein led to nuclear cataract in mice.[105] On the basis of solution experiments, it appears that once a disulfide bond forms at one site it can be transferred to other crystallin molecules.[106]

Chaperones

Based on the presumed role of α-crystallin in delaying the processes that lead to lens barrier formation, it is possible that in other tissues it and other chaperones could play important roles in sequestering altered LLPs. It is well known that modified proteins are more susceptible to cellular proteolysis.

Although it will not be discussed in detail in this review, racemized and cross-linked proteins, as well as those covalently linked to reactive metabolites, generally cannot be processed by the cellular enzymes present in the proteasome or in lysosomes. In cells other than the lens, this will, over time, result in the accumulation of protease-resistant fragments. Once this nonbiodegradable proteinaceous material reaches a certain level, normal metabolism may be impaired. As discussed elsewhere in this review, peptides accumulate in the aged lens primarily due to spontaneous peptide bond cleavage. The chaperone literature is huge, so for more information, the reader is referred to two recent reviews (Refs. 107 and 108) concerning small heat shock proteins, such as α-crystallin.

No Animal Model for ARNC

Once it was recognized that gradual deterioration of macromolecules in the human lens over decades is responsible for ARNC, it became clear that there are no appropriate short-lived animal model systems for studying ARNC.[109] This revelation came as a shock to many researchers trained in the well established, and historically successful, animal-based strategy of elucidating other disease processes. This traditional approach could be paraphrased: detect a suspect protein and gene for the disease, modify/delete it/overexpress it in an animal model to see the effect, and then try to modify the outcome.

The conclusion that experiments using short-lived animals are not likely to uncover the etiology responsible for ARNC does not rule out the use of animals entirely. Long-lived primates remain as one option. It is also probable that the mechanisms of some individual processes, for example, oxidation, can be studied validly using guinea pigs exposed over time to, for example, hyperbaric oxygen.[110] Guinea pigs are most suited to this since they, like humans, lack the ability to synthesize vitamin C.

If, indeed, the use of animals, or animal lenses, are unlikely to yield much information of use for elucidating the events that are responsible for ARNC, what approaches are valid?

In Vivo Assessment

One approach could involve the use of in vivo techniques to assess lens aging as well as early stages of human cataract and

its progression. Dynamic light scattering is one such method, for example,[111] and it could be coupled with the more established approaches such as the Lens Opacities Classification System.[112] This strategy has the distinct advantage in that it is noninvasive, can be used to follow changes over time, and could be particularly useful for assessing the effectiveness of drug therapies.

Signals that may correspond to macromolecular degradation can be measured and correlated with the degree of lens opacity, see for example, de Castro et al.,[113] Besner et al.,[114] and Datiles et al.[111] While such in vivo approaches can provide valuable time profiles, they typically provide a measure of just one of many factors discussed above that could underpin opacification.

The Future of ARNC Research

Primate Lenses

From the foregoing sections, it is evident that the most appropriate animal models for studying ARNC are long-lived primates, for example, chimpanzees. Aside from the expense of carrying out the very long-term studies necessary to replicate age-induced changes to lenses, there are ethical issues. Since longitudinal studies are already underway using primates for other reasons, ocular investigations could presumably be piggybacked to obtain lenses extracted from animals of known age to determine to what extent chimpanzee lenses are similar to human lenses.

In most cases, intact human lenses seem to offer the most relevant tissues for studying ARNC. Lenses of known age and medical history are available from eye banks. In relation to this, it should be recognized that lenses of teenagers and younger individuals available for use in research are often rare. Given the ubiquity of cataract surgery and intraocular lens implants, it is also now very difficult to obtain intact cataract lenses.

The Lens Barrier and Membrane Research Projects

Although it has been shown that by middle age the intralenticular movement of water and small metabolites such as GSH is impaired in normal lenses,[20,21] it will be important to determine whether a more restricted flow from cortex to nucleus is observed in ARNC lenses. If the lens barrier is critical for subsequent development of ARNC, is it possible to mimic the onset of the barrier using isolated lenses or other tissues and show that binding to the interior surfaces of cells does cause impairment of cell-to-cell transport? In relation to this, what is the principal mechanism of attachment of aggregates to the inner cell membrane surface? Can this be disrupted by small molecules? If so, this may lead to new drug leads for potential use in cataract prevention. It is likely that occlusion of membrane pores, such as the connexins and aquaporin 0, by the protein aggregates underpins the lens barrier, since these channels are responsible for the cell-to-cell movement of water and small metabolites. Do these channel proteins participate directly in the binding? This is not known. It should also be recognized that although the unique composition of human lens membrane lipids[115] means that they are resistant to oxidation, they may be implicated in direct reaction with cytosolic aggregates. These are areas of active investigation.

Can ARNC Be Prevented or Delayed?

From the foregoing discussion, it seems unlikely that ARNC can be prevented or cured using dietary or pharmacologic means. Even if drugs were developed or chaperone mimics synthesized that prevented aggregation or barrier formation, could they diffuse into the center of the lens where they would be required? Such compounds would presumably have to be administered prior to incipient cataract. Laser ablation at the barrier zone could potentially delay the onset of ARNC by creating channels for metabolite entry and egress, however this has not been tested and would need to be performed such that vision were not impaired and the long-term function of the lens maintained.

Possible strategies for cataract prevention have been summarized by Pescosolido et al.[116] The part played by small molecules in the prevention or causation of cataracts, including those associated with diet and steroid drug therapy, have been reviewed (see Ref. 117).

Two papers published in 2015 showed that lanosterol and compounds related to this sterol, which interfere with protein aggregation, could act as potential anticataract agents.[118,119] Although activity against animal cataracts was demonstrated, the caveats mentioned in relation to the aged human lens may still apply. This is an ongoing area of investigation with possible promise to develop a range of novel therapeutics. The potential of such molecules to provide a nonsurgical treatment for cataract received a setback when detailed investigations failed to provide evidence that lanosterol or 25-hydroxycholesterol have anticataractogenic activity or bind aggregated lens proteins to clarify cataracts.[120] It is probably not possible to intervene in the spontaneous racemization, deamidation, or truncation events that characterize aged lens proteins since these appear to be driven primarily by heat and time.[16] On the other hand, it may be feasible to reduce protein-protein cross-linking since the dominant reaction involves nucleophilic addition of Lys or Cys residues.[88,90,92,121] Administration of small molecules that contain, for example, amine groups could potentially compete with cross-linking. Naturally occurring molecules such as sarcosine and taurine, or modified homologs, are potential options.

RNA and Epigenetics

One microRNA (miR-34a) plays a part in the regulation of tissue senescence. When miR-34a expression in lens epithelial cells obtained during cataract surgery was measured, it was discovered that miR-34a expression not only increased with age at the time of surgery, but it also correlated with the severity of age-related cataract.[122]

The recently discovered Klotho gene family consists of three members that encode type I transmembrane glycoproteins with extracellular β-glycosidase-like domains. Expression of these proteins appears to be a factor in the progression of age-related diseases in mammals. Of relevance to ARNC, this gene family seems to be involved in the development of cataracts, since differential epigenetic patterns in the DNA around these Klotho genes were found in senile cataract patients.[123]

Correlation as the Conclusion

It should be apparent that while a great deal has been learned about the biochemical processes in the human lens that change with age and with cataract, much of what is known regarding the functional consequences is largely correlative. This is certainly a limitation, but it is one that appears to be unavoidable.

To a large degree, being left with a correlation is a direct function of the time course of macromolecular degradation as well as the broad spectrum of molecular changes to the proteins that take place. It is simply impossible to properly model this vast array of modifications. To exemplify this

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dilemma, one can illustrate the fundamental issues using just one crystallin. In the case of γS-crystallin, the human amino acid sequence is different from that in other animals, and its synthesis alters during our lifetime. With age, it undergoes progressive covalent cross-linking,[57] cleavage,[57] and deamidation[35,124,125] at several sites. Deamidation of Asn 76 may play a crucial role in cataract formation.[35] Some of the γS-crystallin becomes insoluble over time, with the insoluble fraction being more highly modified.[94] Similar caveats apply to the other lens crystallins. Other cellular macromolecules such as membrane proteins, for example, aquaporin 0, and cytoskeletal proteins also degrade in a time-dependent manner. To complicate matters further, cell membrane lipids themselves undergo major age-related modifications.[126]

Lifestyle Factors That May Affect the Incidence of Nuclear Cataract

Epidemiologic data indicate that all people will eventually develop ARNC.[127] Since depletion of α-crystallin appears to be a trigger for the processes that ultimately lead to ARNC, the timing of this chaperone loss may be important in determining the clinical time course of lens opacification. The exact steps whereby loss of free α-crystallin leads to the barrier and oxidation in the lens interior and ultimately to cataract remain to be elucidated.

Temperature

It is not known if there are significant differences in the amount of α-crystallin in the lenses of individuals at birth. The rate of protein denaturation in the lens will very likely influence the time of cataract development. Aside from the relentless spontaneous modifications outlined earlier, temperature could also be a major driver of crystallin denaturation. The temperature of the eye is lower than that of the body—about 35°C. Despite this feature, over a period of thousands of hours some unfolding of proteins is inevitable.

Interestingly, the temperature of the eye, and the lens in particular, can change to a marked degree in response to the environment. For example, the lens temperature of a monkey exposed outdoors in the sun to an ambient temperature of 49°C rose to 41°C within 10 minutes.[128] In the absence of intimate blood contact, the closest source being the retina, this steep increase in lens temperature is driven both by light absorption by the iris and a relatively large surface area of the cornea that is exposed to ambient temperature. Conversely, housing rabbits at 4°C resulted in their lens temperatures dropping by 7°C.[129] If, as predicted, temperature is a major driver of protein denaturation in the lens, this huge ocular temperature range of more than 12°C would have significant consequences.

It is clear that lifestyle can significantly determine lens temperature. With this reasoning, saunas should be of particular interest to cataract epidemiologists, as could other internal factors that affect body temperature, for example, fevers. It is already well known that glassblowers, foundry workers, and blacksmiths are prone to cataract due to years of exposure to infrared radiation,[130] although these cataracts are predominantly posterior subcapsular.

If ambient temperature over the lifespan were to have clinical consequences, one prediction would be that those who live in cooler climes may show a delayed onset of cataract presentation compared to those in the tropics. Although this will be influenced by other factors such as genetics and lifestyle, this prediction is borne out by some epidemiologic data.[131] Temperature is typically not considered in the interpretation of UV exposure data within epidemiologic studies, where it could be a confounding variable.

Other factors act in concert with the thermal unfolding of proteins. Principally, these involve the decomposition of susceptible amino acids, since spontaneous processes occur more rapidly at elevated temperatures. Individual reactions were summarized earlier in this article.

Diet and Lifestyle

As outlined above, lifestyle and environment could play a part in influencing the inexorable decay of lens proteins. Epidemiologic studies support a role for diet in modifying the incidence of age-related cataract.[132] This aspect was also reviewed (see Ref. 133). A higher intake of protein, vitamin A, niacin, thiamin, and riboflavin is associated with reduced prevalence of nuclear cataract.[134] Vitamin C intake had a marked effect on the incidence of ARNC.[133]

On the other hand, smoking has been shown in several studies to lead to an earlier likelihood of ARNC.[135] The toxic chemicals present in smoke may conceivably cause lens proteins to decompose to a greater degree, but transposing other lifestyle data into the biochemical realm of the human lens in so far as it influences ARNC causation is extremely difficult. Interestingly, in an English study, weight at 1 year correlated inversely with ARNC.[136] This time of life corresponds approximately to that when the barrier region is being synthesized, implying that structural or biochemical defects within this lens region at a very early age may predispose people to ARNC many decades later.

Gaps in Our Knowledge of ARNC Etiology

It is now clear that all aged normal human lenses are composed of many highly modified proteins. Despite this plethora of age-related spontaneous modifications, such lenses can remain transparent. It is remarkable that, given the great genetic variability that characterizes the human population, patients with ARNC display characteristic biochemical features that track closely with the degree of cataract. This supports the view that an underlying process is responsible for ARNC. Some have argued that oxidation may be a key event responsible for turning an old, highly modified lens into an opaque cataract lens.[5] The high degree of correlation of protein sulfhydryl oxidation and methionine sulfoxide content with ARNC progression is one factor that supports such a contention.[66–68,137]

There remain a number of features that need to be investigated to enable us to link the various aspects of ARNC formation. Some are listed below in the form of questions.

Since there are many age-related modifications to proteins in the human lens, including deamidation, racemization, truncation, UV filter modification, ascorbate/glucose modification, methylation, and cross-linking, which PTMs are of most importance in inducing protein insolubility? What is responsible for the large-scale attachment of crystallin aggregates to lens fiber cell membranes at middle age? As noted earlier, is the impediment to diffusion of GSH or water at the barrier greater in ARNC lenses than comparable age-matched normal lenses? How does the proposed water circulation model within the lens[138] relate to barrier formation at middle age?

In the vast majority of cases, no time course data are available that could provide a kinetic analysis of all the individual processes. For example, once the lens barrier develops at middle age, does it become progressively less permeable with time? How long does it take for the center of such a lens to become an oxidative environment? What factors are chiefly responsible for this, and to what concentration/flux

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does GSH need to drop in order for the vast oxidative changes to proteins to take place? Does oxidation of these crystallins occur rapidly once GSH decreases below a certain crucial level? In summary, there are many questions that need to be answered before a comprehensive understanding of ARNC etiology is attained.

The Importance of Careful Lens Dissection

In all advanced ARNC human lenses, nuclear levels of GSH are zero, or close to zero. It is very important to differentiate whole lens data from that in which the nucleus has been carefully dissected from the cortex. This is because, as stated in this review, even if nuclear GSH levels fall to zero in advanced cataract lenses, the cortical GSH levels can still be normal.

Other Human Age-Related Diseases and ARNC

Can Cataract Serve as a Model for Other Human Age-Related Diseases?

Due to our increasing longevity, humans are now subject to an increased range of age-related diseases. A key question is this: Do the molecular events that precipitate ARNC exemplify features that apply to other human age-related diseases?

It is likely that this is indeed the case, due to the fact that lens proteins are not the only LLPs in the body. In fact, there are many LLPs distributed throughout the body. The brain, in particular, harbors many such LLPs.[139,140]

Data are not extensive at this stage but it is clear that all LLPs suffer some degree of deterioration with age. This is true, for example, in elastin from the human lung,[141] heart, and arteries[142] and to collagen from a range of tissues.[139,143–145]

Brain Proteins and Lens Proteins Both Deteriorate With Age

The brain may well be the most important of all organs for elucidating the part played by repair or putative ameliorating pathways because there is no prospect of surgical replacement. To exemplify the link between LLPs in the brain and LLPs in the eye, data for two brain polypeptides: myelin basic protein (MBP) and amyloid β (Aβ) will be summarized below.

Multiple Sclerosis and ARNC

Myelin in the central nervous system is laid down during childhood, and thereafter, based on proteomic and racemization data, there seems to be no turnover of the major protein components, for example, MBP. There are many parallels between the age-dependent deterioration of MBP in the brain and those of crystallins in the lens. A large proportion of Asp in MBP was discovered to be present in adults as a mixture of D-Asp and D- and L-isoAsp isomers.[146,147] The fact that nerve conductance and the operation of the brain continues despite large-scale degradation of axonal insulation is itself surprising. In this regard, it is analogous to the adult lens, which remains transparent despite a vast amount of PTM. One notable difference between the brain and the lens proteins is that a number of Arg residues in MBP are found as citrulline—probably due to the action of arginine deiminase.

In the cerebella of multiple sclerosis patients, MBP was found to have been degraded to a significantly greater extent at many sites[146] than was MBP from unaffected individuals. It has been proposed that this site-specific modification of MBP could be responsible for initiating the autoimmune response that is recognized clinically as multiple sclerosis.[148]

Dementia and ARNC

Despite decades of intensive research, there are still no effective treatments or efficacious drugs to cure or even slow the progression of Alzheimer disease (AD). The same is true of the other dementias that afflict the elderly. In AD, data indicate that the peptide Aβ is the key molecule involved in initiating the disease.[149,150] Aβ is very prone to aggregate and most is present in the brains of AD patients as extracellular amyloid plaque. When AD plaque was analyzed, the vast majority was found to have been modified in the same manner as described above for proteins in aged lenses.[151,152] For example, Asp residues were present as isoAsp,[149] a large proportion of Aβ was cross-linked,[153] and D-Ser was found at several sites,[154] as was cleavage of N-terminal to Ser. In addition, the majority of plaque was found to be composed of truncated (laddered) versions. These various modifications could be reproduced by incubating sequences of the Aβ peptide, showing that these products were the result of spontaneous reactions.[62] Amyloid plaque is thus highly heterogeneous, and it remains to be determined how these multitudinous, closely related forms interact to cause the disease and to what extent animal models will play a role in the elucidation of AD.[155]

Overall Summary

A great deal is now known about the aging of the lens and the reactions that are ultimately responsible for ARNC formation. Sadly, it is not likely that this depth of knowledge will enable us to cure cataract. There remains some hope that drugs or surgical procedures may allow the inevitable progression toward cataract to be slowed.

Of wider significance, information gleaned from the study of age-related protein degradation in the lens and its consequences will in the future prove invaluable in understanding a range of other human diseases associated with aging. This is particularly true for the "signatures" of protein degradation. Having identified susceptible amino acid residues, as well as the products of their deterioration in lens proteins, researchers in other fields will be able to determine the extent and location of age-related protein degradation in other tissues. A combination of proteomics and immunohistochemistry, using antibodies raised to these unique chemical structures of degraded amino acids, should be fruitful in determining the sites and extent of protein breakdown in cells from other organs.

In the case of cataract, a simple surgical procedure involving lens replacement can restore vision. Such a straightforward cure is unlikely to be available for other age-related diseases. This is especially so for conditions that result from deterioration of LLPs in the brain. For this reason, it is crucial that the various pathways of LLP degradation continue to be characterized and processes that can influence these in the body be identified.

Characterizing the molecular basis for ARNC may be viewed in the future as a fundamental stage in understanding general processes that are involved in a range of debilitating human diseases of old age.

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