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Selenite Nuclear Cataract: Review of the Model

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Selenite overdose cataract, an experimental model of nuclear cataract produced in young rats is reviewed. Topics include procedures for cataract production and assessment, metabolic and molecular changes in the epithelium of the lens, calcium accumulation, activation of calcium activated protease system, mechanisms for crystallin precipitation, anti cataract drug testing and relevance to human cataract..

Selenite-overdose cataract is an extremely rapid and convenient model of nuclear cataracts. The cataract is produced in suckling rat pups by an overdose of the essential trace mineral selenium (Figure 1). The ability of selenite to cause cataracts was first seriously described in 1977 (1). Numerous journal articles concerning the cataract have been published since then. Previous reviews focused on fundamental biochemical mechanisms causing selenite cataract (2, 3) and on the use of selenite cataract in the elucidation of the calcium-activated protease pathway in rodent cataract formation (4). The purposes of the present review are to update these mechanisms, to include new molecular biology data from the five year period 1992-1997, and to address the current use of selenite cataract as a model for testing anti-cataract drugs.

Procedure for Producing Selenite Cataract and Developmental Stages— Selenite cataract is usually produced by a single subcutaneous injection of 19-30 µmoles/kg body weight of sodium selenite (Na_2SeO_3) into suckling rats of 10-14 days of age (See Appendix on "Selenium Safety Concerns" and "Selenium Disposal)." Control injections of sulfite (SO_3) at the same oxidation state as SeO_3 did not cause cataracts (5). Thus, uninjected animals can serve as controls. m-calpain (millicalpain, EC 3.4.22.17, also termed calpain II) is a major proteolytic enzyme in lens involved in selenite cataract formation (4). Moreover, repeated injections of smaller doses of selenite (6) or oral administration (7) are also cataractogenic. Selenite is cataractogenic only when administered to young rats before completion of the critical maturation period of the lens (approximately 16 days of age). Severe, bilateral nuclear cataracts are produced within 4-6 days. Precursor stages include: posterior subcapsular cataract (day 1), swollen fibers (day 2-3), and perinuclear refractile ring (day 3). Although the model has been used extensively as a model for nuclear cataract; a transient cortical cataract also forms 15-30 days after injection (4). The cortical cataract then clears after several months, but the nuclear cataract is permanent. The cortical cataract has been well characterized histologically and involves protein degradation, liquefaction, and abnormal fibergenesis. However, this cortical cataract is not as convenient as the nuclear cataract because of the longer development time. Furthermore, because of abnormal fibergenesis, selenite cortical cataract is not a clear cut model of a calpain-induced cataract as is the rapidly forming selenite nuclear cataract. This review focuses on selenite nuclear cataract.

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Mechanisms of Selenite Nuclear Cataract— Several biochemical processes occur during production of selenite cataract (Figure 1). These include: altered epithelial metabolism, calcium accumulation, calpain-induced proteolysis, crystallin precipitation, phase transition, and cytoskeletal loss.

Altered Metabolism in Lens Epithelium— A number of important changes in metabolism have been documented in lens epithelium during formation of selenite cataract, usually well before any visible opacity. These include suppression of mitosis and entry of epithelial cells into prophase, nuclear fragmentation (8), decreased rate of epithelial cell differentiation (9), decreased synthesis and increased damage of DNA (10), and loss of calcium homeostasis (11). Loss of calcium homeostasis could be prevented by antioxidants. We hypothesize that these early changes in lens epithelium may result from oxidative damage caused by selenite, possibly to critical sulfhydryl groups on molecules such as calcium ATPase or ion channels.

More recently, epithelial cell death (necrotic or apoptotic) has been proposed as contributing to formation of cataracts (12). Our preliminary data also indicate that epithelium from rat lenses cultured in A23187 show an increased terminal deoxytransferase-mediated dUTP nick-end labeling (TUNEL) assay (increased fragmented DNA). This suggested apoptosis in these cultured lenses with increased calcium, which is also observed in selenite cataract. Other factors involved in cell death include breakdown of cytoskeletal proteins, such as alpha-spectrin. Selenite cataract shows extensive breakdown of alpha-spectrin (13). Calpains and other proteases, such the interleukin-1 ß-converting enzyme (ICE) related proteases, are involved in cell death in other tissues (14), and these might be activated after the oxidative changes caused by selenite injection. These extensive changes to the epithelium undoubtedly influence some of the other changes in selenite lenses described below.

Calcium Accumulation— Most cataracts show an increase in lens calcium (3). Selenite cataract is especially interesting because calcium uptake during nuclear cataract formation is highest in the nucleus, and the calcium concentration in the cortex remains lower during this period (15). In contrast, sodium, potassium, and water do not increase (16), indicating no appreciable changes in generalized permeability of the whole lens. Rapid changes in dry mass have been found in regions surrounding the nucleus, suggesting that rapid changes in water distribution can occur in specific regions of selenite cataract (17). Theoretically, increased lens calcium could be due to inhibition of outwardly directed Ca-ATPase pumps or inhibition of Na/Ca exchange. Lenses from rats injected with selenite showed a 50% decrease in Ca-ATPase activity (11). Furthermore, selenite did not inhibit Na/K ATPase in cultured rabbit lenses (18). A recent study using lenses from rats injected with selenite found that inhibition of Na/K exchange was not a major factor in calcium accumulation (19). Taken together, these data indicated that inhibition of Ca-ATPase may be the most important mechanism for calcium accumulation in selenite nuclear cataract. Thus, increased lens calcium may be due to oxidation of sulfhydryls and to other changes in the membranes caused by selenite, leading to inhibition of the Ca-ATPase pump and selective calcium permeability. The intriguing question as to why the nucleus shows such markedly elevated calcium is still unanswered, but it may be related to the immaturity of suckling rat lens used in selenite cataract, such as open sutures (4).

Proteolysis— An important consequence of calcium elevation in rodent lenses is activation of the calcium activated protease, m-calpain (Figure 2). m-calpain is a major calcium-activated non-lysosomal, cysteine protease in rodent lenses. Lenses from human (20), cows (21), mice (22), rats (23), rabbits (Fukiage, Azuma, and Shearer, unpublished data), and guinea pigs (22) all contain m-calpain. Several lines of evidence have been used to provide convincing evidence for calpain activation in selenite cataract. Crystallins in selenite cataract were compared to isolated lens proteins incubated with purified calpain (24). The truncated crystallin polypeptides produced in both cases showed similarities. 1) The migration patterns of the truncated crystallins observed with sensitive two dimensional electrophoresis were similar (25). 2) At least eight of the new proteolytic fragments from ß-crystallins from the in vivo and in vitro experiments have the same calpain-like cleavage sites on their N terminal extensions (Figure 3) (24). 3) The in vivo precipitation of ß crystallins occurring in selenite cataract can be mimicked by addition of calpain to total soluble proteins from normal rat lens (Figure 4). Studies using similar experimental design and methods indicated that calpain induced proteolysis is a common underlying mechanism for a wide variety of cataracts in rats and mice. These included those induced by buthionine sulfoximine, calcium ionophore A23187, hydrogen peroxide, diamide, xylose, galactose, streptozotocin and Nakano genetics (26, 27). Thus, regardless of the cataractogenic factor initiating calcium entry, the calpain-proteolytic mechanism in cataract formation seems to be very active in rodent lenses.

Thus, very strong evidence, especially from selenite cataract, shows that activation of calpain is a common underlying mechanism in many rodent cataracts. Caution needs to be exercised in applying this mechanism to other situations. Situation 1 - Normal rat lens maturation: During normal maturation of rat lens, more than 50% of the proteins become water-insoluble (urea soluble) when homogenized (28). Many of the soluble and insoluble ß-crystallin polypeptides contain calpain-like cleavage sites on their N-termini. This is an important observation because it means that calpain is activated even during normal rat lens maturation, where calcium concentrations are not obviously elevated. Note that in vivo these lenses are transparent. Thus, calpain proteolysis does not always cause opacity, especially if the proteolysis is slow as in normal lens maturation. Situation 2 - Cortical opacification: Cleavage site analysis also shows that ß-crystallins in the cortex of rat lenses cultured in calcium ionophore undergo calpain-induced proteolysis (29). Large

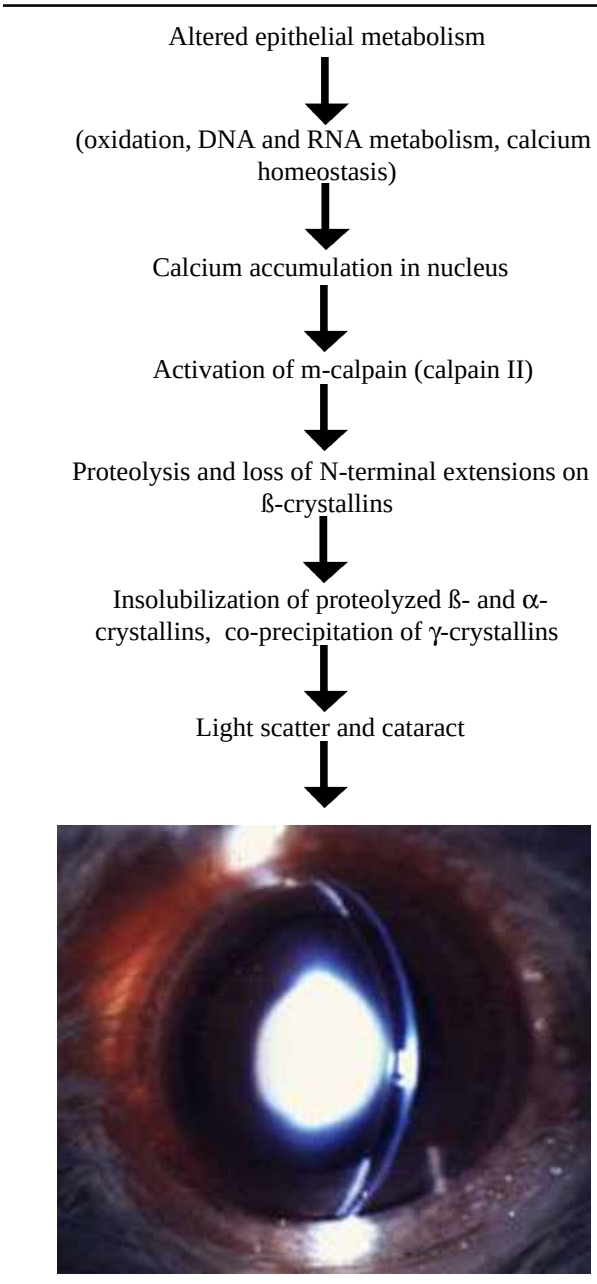

Figure 1. Mechansim of selenite nuclear cataract formation.

amounts of insoluble protein are produced, and cortical opacity is evident. However, the opacity cannot be prevented by calpain inhibitor E64. This means that the opacity may not be directly caused by calpain proteolysis. In this case, calpain proteolysis co-exists with cortical opacity. Situation 3 - Proteolysis in non-rodent lenses: The major proteins in young human lens have now been completely identified (30, 31) by combining two dimensional electrophoresis, automatic Edman protein sequencing of spots from the 2D-gels to provide actual amino acid residues at cleavage sites, and mass spectrometric analysis to determine the molecular weight of crystallin fragments. However, to date, such analyses have found only minimal amounts of naturally-occurring, calpain-like cleavage sites in bovine lenses and none in crystallins from human and chicken lenses. (David, Lampi, Shih, and Shearer, in preparation). This was unexpected because crystallins from all three species are substrates for calpain in vitro (32). These lenses do show accumulation of truncated crystallins with age. This means that other proteases or non-enzymatic processes are responsible for the breakdown of crystallins in non-rodent lenses, or that another protease(s) working in combination with calpain obliterate calpain-like cleavage sites in aging human, bovine and chicken lenses. The proteases causing cleavages in these non-rodent lenses are unknown. On the other hand, the data above from the selenite cataract clearly indicate that calpain causes rapid proteolysis, precipitation of lens crystallins, and cataract in young rodent lenses. Studies described below are underway to understand the mechanism of calpain-induced precipitation in these young rodents in the hope that this will provide a foundation for understanding crystallin precipitation in man and other non-rodent models.

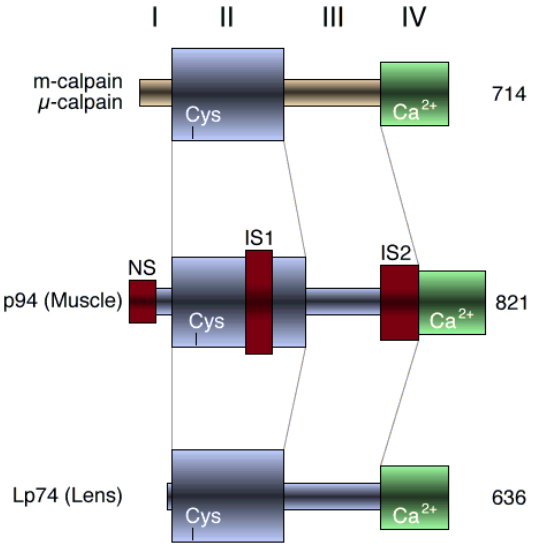

Figure 2. Domains of calpain isozymes. Schematic domain structures for m-calpain and μ-calpain compared to tissue-type calpains found in muscle (p94) and rat lens (Lp74). The Roman numerals designate domains I (autolytic), II (cysteine protease catalytic site), III (unknown function), and IV (calmodulin-like calcium binding regions) for the family of calpains. NS (novel sequence), IS1 (insert 1) and IS2 (insert 2) in red are sequences unique to p94 and deleted in Lp74. The ubiquitous m-calpain and μ-calpain also have domains I-IV, but are separate gene products showing 44-49% homology to p94 and Lp74. Numbers to the right are total amino acid residues in the primary sequences.

Formation of Insoluble Protein in Selenite Cataract— The nature of the forces promoting formation of the insoluble pellet in the rapidly forming, selenite nuclear cataract may be unusual compared to other cataracts forming over a longer time period. The pellet in selenite cataract is obviously massive, comprising over 17% of the total protein in the lens nucleus of the young rat (33). The tendency is to view such cataractous pellets as covalently-linked, high molecular weight aggregates of crystallins. However, currently there is no evidence for covalent associations between crystallins or the truncated crystallin polypeptides within the pellet from selenite nuclear cataract. No new protein-protein disulfide bonds (Prot-S-S-Prot) are found in the pellet (34); no high molecular weight aggregates in the soluble alpha-fraction or above have been isolated on gel chromatography (35). In fact, the most notable observation with gel chromatography is a shift from the ßH-crystallin to the ßL-crystallin fraction, probably related in part to high susceptibility of ß-B1 polypeptide to proteolysis by m-calpain. The pellet from selenite cataract is not dissolved by reasonable changes in pH or addition of salt (25). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE) of the insoluble fraction of selenite cataract shows loss of higher molecular weight crystallins and non-crystallin proteins, with no evidence of calcium-activated transglutaminase cross-linking. Thus, the pellet formed in selenite nuclear cataract should be viewed as crystallins and the truncated crystallin polypeptides which have simply become insoluble. Recent studies on in vitro precipitation of rat lens crystallins by calpain and studies on phase transition have tended to support this view.

In Vitro Model of Crystallin Precipitation— Strong support for the role of m-calpain in precipitation of crystallins in selenite cataract has been provided by an in vitro model of proteolytic

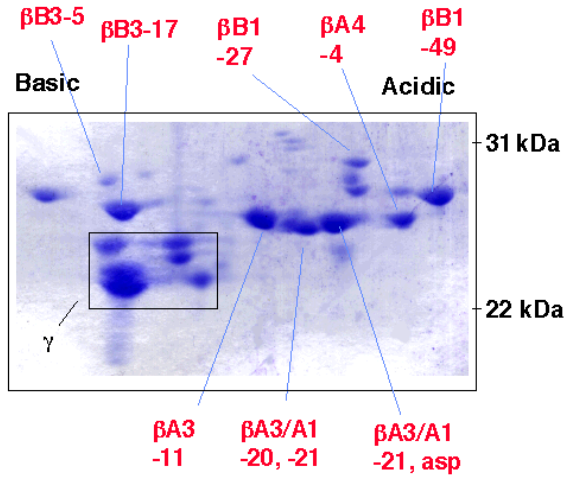

Figure 3. ß-crystallins proteolytic fragments from selenite nuclear cataract. Cleavage sites on the N-terminal extensions of ß-crystallin polypeptides in the insoluble pellet from selenite nuclear cataract. This figure identifies only those polypeptides in selenite cataract showing the same cleavage sites as found in ß-heavy crystallins incubated with m-calpain in vitro (24). The data were obtained by blotting and N-terminal sequencing the protein spots from two-dimensional electrophoretic gels. (Truncated αA spots are missing from this gel.)

precipitation. Large amounts of purified m-calpain at 7 U/mg protein were added to soluble proteins isolated from the normal young rat lens (35). Within 20 minutes after activation of the calpain with calcium, massive precipitation of proteins occurred. The insoluble pellet consisted mainly of truncated, insolubilized ß-crystallin polypeptides. The insolubilzed ß-crystallin fragments contained many of the same calpain cleavage sites as in selenite cataract (24, 25). These cleavage sites were on the N-terminal extensions, and from 4 to 49 amino acids were missing, depending on the ß-polypeptide. These data suggested that proteolysis of the N-terminal extensions causes truncated ß-crystallins to rapidly insolubilize. Even when physiological concentrations of 120 mM KCl were added to the incubation mixture to minimize ionic interactions between ß-crystallin fragments, massive light scattering occurred. As in selenite cataract, calpain induced light scattering was attenuated by aging (Figure 4).

To more closely mimic the situation in selenite cataract, the above in vitro model has been improved recently by using the endogenous calpain in young rat lens. Activation of endogenous calpain by adding calcium caused rapid proteolysis of α- and ß-crystallins, but a considerable lag period of 5-7 days occurred before precipitation of truncated crystallins (25). The precipitated pellet again contained truncated of ß crystallins with calpain-like cleavage sites on their N-termini. However, the overall pattern of precipitated polypeptides in this endogenous model as observed with two-dimensional electrophoresis more closely resembled the pattern observed during in vivo selenite cataract (25). That is, the pellet also contained truncated α-crystallins and large quantities of intact γ-crystallin in addition to truncated ß crystallins. This indicated that the loss of solubility of ß-crystallins induces co-precipitation of intact γ-crystallins. We have also found that long term calcium potentiates, but is not required for calpain induced precipitation (Mizuno and Shearer, unpublished data). In other words, if calcium is present for activation of calpain, truncated polypeptides are produced and these precipitate without the necessity of calcium. Since KCl was present to minimize ionic interactions, the loss of the N-terminal extensions on ß-crystallins may have caused precipitation by promoting loss of water associated with N-terminal extensions, exposure of hydrophobic residues on the remaining globular regions, or dimer rearrangement. These processes could lead to abnormal, associations between ß-dimers which are then more insoluble. Or the loss of the N-terminal "spacer" extensions between ß-crystallin dimers could cause precipitation. Future computer modeling of truncated versus intact crystallin subunits may help determine which of these mechanisms causes truncated ß-crystallin fragments to become insoluble.

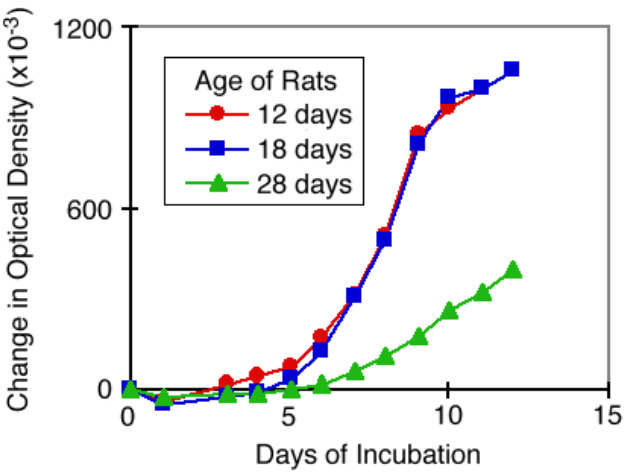

Figure 4. Opacification of lens proteins by m-calpain. In vitro light scattering in the lens soluble proteins from rats of 12, 18, and 28 days of age caused by incubating with m-calpain (Shearer and Shih, unpublished data). Note that this in vitro effect is attenuated with age as occurs with the selenite cataract. The vertical axis is change in optical density at 405 nm.

Another factor influencing precipitation in this model, as well as selenite cataract, is the concurrent calpain-induced proteolysis of α-crystallin. Both the A and B chains of α-crystallin are substrates for purified m-calpain in vitro and for endogenous m-calpain in selenite cataract (36). In contrast to ß-crystallin, calpain causes the loss of the C-terminus of α-crystallin polypeptides. This is important because the loss of the C-terminal peptide from α-crystallin may induce conformational changes and reduce chaperone activity (37). Truncated α-crystallins from in vitro incubation of intact α-crystallin with m-calpain, or from selenite cataract, show decreased chaperone ability (36). Decreased chaperone ability would promote formation of insoluble protein in selenite cataract. All the preceding studies were performed with lenses from rats. However, we recently found that incubation of total soluble proteins from young mouse lens with purified calpain or activation of endogenous calpain in mouse lens caused precipitation of crystallins (22). Thus, the in vitro incubations with calpain closely mimic selenite cataract and lend strong belief to calpain-induced, proteolytic insolubilization as a mechanism of cataract formation in certain rodent models.

Interestingly, μ-calpain (also termed calpain I) was also able to cause in vitro precipitation of lens crystallins like m-calpain (38). This isoform of calpain is found in very low levels in lenses (21). μ-calpain is of interest because it requires approximately 4 μM calcium for half maximal activation which is much lower than the 200-500 μM calcium required by m-calpain. μ-calpain is probably not physiologically significant in selenite nuclear cataract because the massive increases in calcium (about 1000 μM) are able to activate the much more abundant m-calpain. However, μ-calpain may be important during normal lens maturation. Calpain proteolysis and insolubilization occur in normal lens maturation (28), under conditions where calcium increases are probably minimal, transient, and localized.

There are important exceptions to calpain-induced insolubilization of crystallins. To date, only crystallins form young rat and mouse precipitate in vitro when proteolyzed by calpain. Total soluble proteins from lenses from the following sources do not show in vitro light scattering when incubated with m-calpain: older rats, older mice, and any aged guinea-pig (another rodent), cow, rabbit, chicken, or human (32, 22). This was unexpected since crystallins from all these sources were proteolyzed by calpain. The in vitro conditions for precipitation of crystallins may not yet be optimal for these other species since these lenses obviously show increased

amounts of insoluble protein with in vivo aging and/or cataract formation. Whatever the reason, truncated ß crystallin polypeptides from young rat and mouse lenses seem especially susceptible to precipitation under our current conditions.

Possible reasons for the above species differences in susceptibility to calpain-induced precipitation include differences in cleavage sites on individual crystallin polypeptides, amino acid differences in crystallins between species, length of remaining truncated polypeptides, or exposure of hydrophobic residues. Other factors such as the higher protein content of intact lenses, and the presence of membranes or cytoskeletal proteins may be necessary. Unique ratios of polypeptides in young mouse and rat lens may promote precipitation. For example, the SDS-PAGE patterns of crystallins from susceptible young mouse and rat lens were similar to each other, but very different from the pattern in resistant guinea pig. ß B1 and ß-B3 crystallins make up a larger proportion (8-11%) of the crystallin polypeptides in young mice and rats than in guinea pigs (3-4%). Detailed two-dimensional electrophoretic mapping and sequencing of normal and calpainized lens soluble proteins may be able to determine why certain truncated crystallins do or do not precipitate. Young rodent lenses appear to provide an unusual opportunity to discover why certain crystallin fragments precipitate. Furthermore, discovery of a non-rodent source of crystallins which would precipitate after addition of calpain or other lens proteases would also have two major benefits. First, it would provide more proteins for analysis than small rodent lenses. Second, such data would show which primary sequences, cleavage sites, protein conformations, polypeptide ratios, or other proteases are most related to propensity for insolubilization and cataract formation.

Phase transition— Recent transmission electron microscopy pictures of selenite nuclear cataract showed formation of a two phase system and aggregation of the cytoplasmic protein onto the membranes (39). These studies added to the early TEM studies on selenite cataract showing precipitation of the cytoplasm (40) by using Fourier analysis to quantify TEM changes (39). The authors found that selenite changes the normally small spatial fluctuations in the protein density in the cytoplasm to spatial fluctuations measuring in the order of the wavelength of light. The results provided a quantitative basis for opacity in selenite cataract.

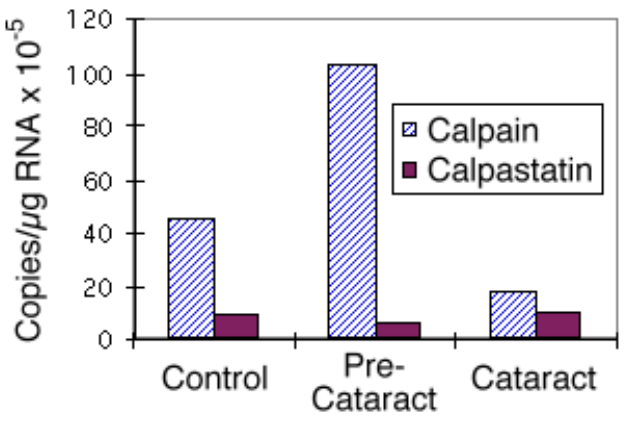

Figure 5. mRNA levels for m-calpain and calpastatin in selenite cataract. The mRNA levels were quantified with competitive RT-PCR using RNA with deletions in mRNAs for calpain (44) and calpastatin. Note the up-regulation of m-calpain transcripts before selenite nuclear cataract formation.

Selenite administration has a complex and unexpected affect on phase transition in rat lens. Two days after selenite, lenses are actually less susceptible to cold cataract (41). The critical temperature for phase transition, Tc, was lower than controls. These lenses were homogenized, and the total soluble proteins were tested for their susceptibility to phase separation. The lens total soluble protein from rats injected with selenite were found to be more susceptible to phase transition (41). This effect has been localized to solutions of proteins from the nucleus (42) and not observed in the cortex. The Tc then sharply increased just before nuclear cataract formation, making the lenses susceptible to cold cataract at room temperature. The early decrease in susceptibility to phase separation has been hypothesized to be related to the increased amino acid content and decreased intracellular volume of lenses observed by 1 to 2 days after selenite injection (5). Thus, phase transition is a well-documented phenomenon in the selenite model. These studies form the basis for using the selenite cataract model to test phase separation inhibitor drugs in cataract prevention (discussed below).

Cytoskeletal Loss— Added to the loss of crystallins and phase separation discussed above, is the loss of cytoskeletal proteins from lenses of selenite-treated rats (33). These losses were recently quantified (13). Selenite accelerated the loss of the cytoskeletal proteins actin, tubulin/vimentin, and spectrin, as well as unidentified nuclear proteins of 49, 60 and 90 kDa. Cytoskeletal proteins, such as the vimentin-actin tubulin system and the beaded filaments, may stabilize the transparent cell structure. The authors hypothesized that calpain-induced proteolysis of the cytoskeletal proteins may disrupt normal interactions between crystallins and the cytoskeleton, leading to opacity. Further studies on changes in the cytoskeletal elements in selenite cataract are relevant for two reasons: 1) cytoskeletal proteins are especially good substrates for calpain in vitro (23), 2) the cytoskeletal proteins may be some of the first proteins lost in selenite cataract (13). Thus, degradation of cytoskeletal proteins may be an important mechanism during the early stages of selenite cataract formation. Experiments on cytoskeletal proteins may be particularly relevant if future studies indicate that calpain is not directly responsible for the crystallin breakdown in inner cortex and nucleus of human lenses. Calpain is higher in the cortex than the nucleus of the human lens (20). Calpain-induced cytoskeletal breakdown in the outer regions of human lens could be occurring but may have gone undetected so far. In older cultured rat lenses, inhibition of cytoskeletal breakdown by calpain inhibitors was associated with reduction in calcium/ionomycin induced, cortical opacity (43). Such data indicate the relevance of studies on cytoskeletal breakdown in early selenite cataract formation.

Molecular Biology of Selenite Cataract— Some of the earliest changes preceding selenite cataract concern DNA and RNA metabolism in the lens. For example, suppression of mitosis and karyorrhexis (nuclear fragmentation) were observed at five hours after selenite injection (8). At 6-12 hours after selenite injection, DNA replication was reduced by 30%, followed by

increased DNA synthesis and repair above control values (10).

mRNA for calpains and calpastatin in lens— Because calpain is important in formation of selenite cataract, mRNAs for the major components of the calpain system (m-calpain, μ-calpain, and calpastatin inhibitor) have been determined in rat lens and in selenite cataract (Figure 5). Methods used included Northern blotting and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using an internal RNA standard containing deletions in mRNAs for m-calpain and calpastatin. mRNAs for both μ-calpain (38) and m-calpain (44) were detected by RT-PCR in normal rat lens. However, the amount of μ-calpain mRNA was much less than for m-calpain. This parallels the protein levels for the two isoforms in rat lens (45). Interestingly, the mRNA levels for calpain II in young rat lens, of $5x10^6$ copies/μg total RNA, were many times higher than age matched rat liver, kidney, lung and brain; and at least five times higher than in young human lens. mRNA levels for calpastatin of $1x10^6$ copies/μg total RNA in young normal rat lens were five times lower than for m-calpain. mRNAs for both m-calpain and calpastatin decreased with chronological and anatomical aging in rat lens. Thus, young rat lens contains an abundance of mRNA for m-calpain compared to mRNAs for calpastatin, μ-calpain, and m-calpain mRNA in other tissues. Such data partially help explain the strong tendency for young rats to exhibit calpain-induced proteolysis after injection of selenite.

Control of the calpain system in selenite cataract was next assessed by measuring changes in mRNAs for m-calpain and calpastatin after injection of selenite (Figure 5). We found that mRNA for m-calpain changed during selenite cataract formation (44). Before nuclear cataract formation, mRNA for m-calpain more than doubled and then decreased by 64% after selenite cataract formation. Amounts of m-calpain protein (immunoreactivity) remained fairly constant during the early stages of cataract formation. These data imply that the control of increased calpain activity during selenite cataract formation is probably at the post translational level by increased calcium. Thus, the significance of the early up-regulation of m-calpain mRNA during selenite cataract formation is currently unknown. We hypothesize that it may be associated with the initial cataractogenic response in the epithelial cells or peripheral cortical fibers caused by injection of selenite.

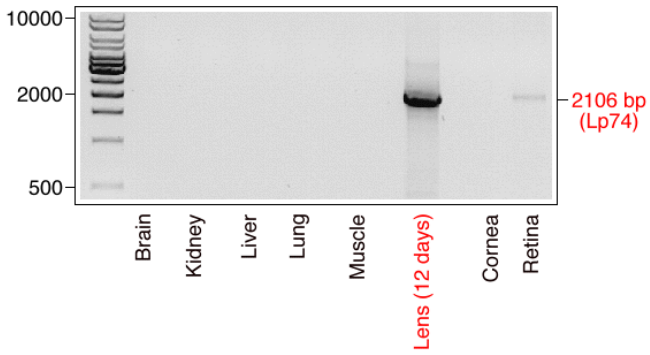

Figure 6. Lp74, a lens specific calpain. mRNA for Lp74 calpain located in lens using RT-PCR with gene-specific primers to full length Lp74. The heavy band at 2106 bp in lens was sequenced and found to be a splice variant of the muscle type p94. In Lp74, most of the nucleotides from exon 1 and all of exons 6, 15 and 16 in the p94 sequence were missing. The primers used for RT-PCR for Lp74 correspond to these sequences in Lp74: sense 167-197, antisense 2243-2270. The cDNA nucleotide sequence and the deduced amino acid sequence for Lp74 are available using GenBank accession number U96367.

mRNA for calpastatin remained relatively constant during selenite cataract formation and aging (Figure 5). Thus, the translation rate of calpastatin mRNA and the long term stability of the calpastatin molecule must effectively inhibit undesirable calpain activity in the lens until calcium levels become fairly high, as in selenite cataract. Total calcium levels in selenite cataract exceed 1000 μM(16). Rat lens m-calpain is 50% active at approximately 250 μM calcium (23). Our recent studies indicate the possibility that m-calpain may hydrolyze alpha crystallin without undergoing autolytic activation when millimolar calcium concentrations are present (46).

Lens-specific Calpains? An exciting recent development, relevant to selenite cataract, is the finding of tissue-specific calpains. For example, compared to mRNA levels of m-calpain, muscle contains a ten fold excess of an mRNA for a calpain termed p94 (47) (Figure 2). Tissue-specific calpains are postulated to have tissue-specific functions not performed by the ubiquitous μ- and m-calpains. (48)

Using RT-PCR with degenerate probes for m-calpain, we recently showed the unexpected presence of "muscle-specific" calpain (p94, also termed calpain 3) transcripts in rat lens (Ma and Shearer, unpublished data). Subsequent RT-PCR using primers specific for p94 showed that, unlike the single transcript in muscle, lens contained two transcripts. One transcript was represented by a PCR product near the expected size for muscle-specific calpain p94. The other product was a smaller, but more abundant transcript now termed Lp74 (Figure 6). Of the eight tissues studied in rats so far, Lp74 was present in significant quantities only in lens. Cloning and sequencing of the cDNA for Lp74 calpain showed that it lacked several exons from muscle type p94. In the deduced protein, this would cause a lack of regions termed NS, IS1, and IS2 unique to muscle type p94 (Figure 2). Thus, Lp74 in lens is likely a splice variant of muscle type p94 calpain. Not only is Lp74 important for lens research, but mutations in p94 cause human limb-girdle muscular dystrophy type 2A (47). We speculate that the sequence of Lp74 may be adapted to the unusual properties of the lens (high protein levels, dehydration, massive influxes of calcium, loss of organelles) to allow unique control of this calpain in lens. mRNA levels for Lp74 are equal to those for m-calpain (Figure 7). Lp74 is only known at the mRNA level. But if Lp74 protein is subsequently found to be expressed in lens, some of the proteolysis in selenite cataract may, in fact, be due to Lp74.

Use of Selenite Cataract to Screen Anti-Cataract Drugs— Selenite cataract has been used for initial screening of potential anti-cataract agents. Staging of selenite cataract for these studies has been performed by slit lamp biomicroscopy with a scale of 0-6 (49), by frequency of characteristic features such as swollen fibers and nuclear cataract (50), and by densitometric evaluation of enucleated lenses using a video

camera attached to a dissecting microscope and computer imaging software (50). Using the slit lamp method for staging, five rat models of cataract were recently compared (49). Selenite cataract was found to be the most reliable and reproducible compared to radiation, galactose, streptozotocin and RCS models, especially for advanced cataract evaluation. The chronic, low dose model of selenite cataract allows administration of drugs over the 10 day period of selenite administration (6). To our knowledge this model has not been used, but it might be useful with slower acting anti-cataract drugs. Furthermore, human nuclear cataracts show lipid membrane vesicles (51). Formation of these cytosolic membrane vesicles was recently used as a measure of the relevance of animal models to human cataract formation (52). Compared to galactose, BSO, and Emory mice, confocal microscopy showed that selenite cataract best approximated vesicle formation seen in human cataracts. Selenite cataract shows a number of general similarities to human cataract in addition to vesicle formation, such as increased calcium, insoluble protein, and proteolysis; and decreased water soluble proteins and reduced glutathione (GSH). A number of major dissimilarities are also present: contrary to human cataract, selenite cataract shows no high molecular weight covalent aggregates or increased disulfide formation, and selenite cataract seems to be dominated by rapid calpain-induced proteolytic precipitation while human senile cataracts may be caused by oxidative stress over a long time period. At this point, the best conclusion as to relevancy of selenium cataract to human cataract may be that selenium cataract is a useful in vivo rodent model for initial drug testing, but that important differences exist between human and selenium cataracts. A number of potential anti-cataract agents have therefore been tested in selenite cataract.

Different drugs were found to have varying levels of effectiveness against selenite cataract, partially because cataract formation involves a number of sequential steps. For example, fourteen different anti-cataract drugs were compared in selenite cataract (53). These included antioxidants, thiophosphates, disulfides, and chelators. Results showed that pantethine (disulfide form of pantetheine) was the most effective and least toxic of the compounds tested. Furthermore, this effect was permanent when administered within an hour after selenite injection. Although the mechanism for this protective effect is unknown, pantethine in vitro does not prevent the direct oxidation of sulfhydryls by selenite (David, unpublished data). The authors proposed that the interaction of pantethine with protein sulfhydryls prevented protein aggregation during selenite cataract formation. Separate studies on individual anti-cataract agents have also included naproxen (a nonsteroidal anti-inflammatory agent), which was 50 to 70% effective in preventing selenite cataract, possibly acting as an anti-oxidant (54). Deferroxamine, an iron chelator, temporarily protected against the early PSC in selenite cataract (55). Thus, the selenite model has allowed initial drug testing in the whole animal as well as offering some insights into the mechanism of cataract protection. Again, investigators must realize the probable differences in human cataract and selenite cataract regarding active proteases, in vitro susceptibility of crystallins to precipitation, and characteristics of the insoluble proteins (reviewed above). However, the reliability and extensive characterization of selenite cataract has probably resulted in the best rodent model so far for rapid screening of potential anti-cataract agents.

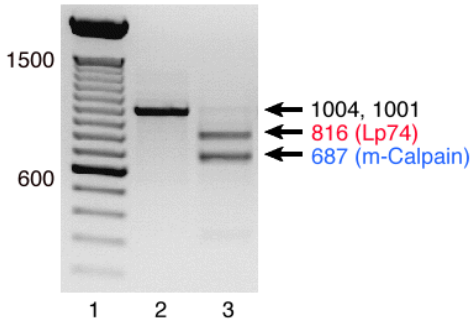

Figure 7. mRNA levels for newly discovered lens calpain, Lp74, compared to m-calpain mRNA using RT-PCR. Lane 1 contains a 100 bp ladder. Lane 2 is RT-PCR using common primers corresponding to nucleotides: sense Lp74 272-292, antisense Lp74 1253-1273; sense m-calpain 415-435, antisense m-calpain 1393-1413. The resulting band in lane 2 contained transcripts for Lp74 and m-calpain not resolved on this gel. Lane 3 contains both Lp74 and m-calpain transcripts cut with BbsI. Note equal amounts of unique bands at 816 bp (Lp74) and 687 bp (m-calpain), suggesting that Lp74 transcripts are as high as those for m-calpain.

Future Directions— The past 20 years have provided a wealth of information detailing the metabolic defects in selenite cataract. We believe these studies provide strong evidence that the nuclear cataract is a well-defined model of cataract produced by calpain proteolysis followed by crystallin precipitation. These studies have also established calpain induced proteolysis as an underlying mechanism in many different types of rodent cataracts. Important future directions for the selenite nuclear cataract include:

1) Test if the proteolytic-precipitation model, as exemplified by calpain action in the selenite cataract, can be generalized to other non-rodent models and human cataract. Is this mechanism important or unique for rodents? Uniqueness seems unlikely in view of the extensive protein breakdown observed in many cataracts from a variety of causes and species. Cleavage site analysis of truncated crystallins from human and non-rodent models of cataract will help determine which proteases are functioning in a particular species. Determine the role of the newly discovered Lp74 calpain in lenses from rat and man.

2) Determine why young rodent crystallins are so susceptible to precipitation after proteolysis by calpain. Conversely, why do crystallins from other species not precipitate even though they are substrates for calpain? Possibly, they would precipitate if proteolyzed by other lens enzymes or incubation conditions. Studies measuring

biophysical changes occurring after proteolysis, measurements of hydrophobicity, and computer modeling of truncated crystallins are needed to determine how proteolysis by calpain and other enzymes renders the truncated crystallins insoluble.

3) Determine if the potential drugs identified as effective against selenite cataract are also effective in other models relevant to human cataract.

APPENDIX

Selenium Safety concerns— Although selenium is an essential trace mineral for man (56), sodium selenite is also highly toxic. The LD_{50} (intravenous) for sodium selenite in rats is listed in the Material Safety Data Sheet (Sigma) as 3 mg/kg. The cataractogenic dose is 2.4 mg/kg. This dose does not cause observable effects in suckling rats, except for occasional skin lesions at the injection site and cataracts. A significant portion of this dose is retained in the carcass because selenium is incorporated into tissue proteins (57). Furthermore, selenium is excreted in urine, feces, and expired air. One litter of rats receiving a cataractogenic dose of selenium is injected with a total of 600 µg selenium. Thus, precautions are needed for animal handlers and for disposal of selenium injection solutions, animal carcasses, and wastes.

Selenium Disposal— Sodium selenite is moisture sensitive and injection solutions must be prepared fresh from the powder for each experiment. A typical injection solution is 10 ml at approximately 3 mg sodium selenite/ml. Most of this is not used since injection volumes in suckling rats are typically small (0.05 ml). The sodium selenite solutions cannot be dumped in the drainage and must be removed by commercial or university chemical hygiene officers for disposal by an EPA-approved disposal facility. Animals are housed in a separate animal room with adequate air turnover. Animal handlers wear gloves, lab coats and surgical masks. At our institution, all contaminated bedding material and carcasses are bagged for subsequent incineration.

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