Regional Differences in Cystine Accumulation Point to a Sutural Delivery Pathway to the Lens Core

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PURPOSE. To develop an imaging technique that maps the distribution of free cystine in the rat lens at subcellular resolution and enables cystine accumulation to be compared with the expression of the cystine–glutamate exchanger (Xc-).

METHODS. Whole lenses were fixed, cryoprotected, and then cryosectioned in either an equatorial or axial orientation. Sections were double labeled with either a cystine antibody designed to detect free cystine (the oxidized form of cysteine) or an antibody against the light chain of Xc-, and the membrane marker wheat germ agglutinin. Sections were imaged by confocal laser scanning microscopy. Cystine labeling was quantified using image analysis software and an intensity profile plotted as a function of distance from the lens periphery. High performance liquid chromatography (HPLC) was used to determine cyst(e)ine levels in the outer cortex, inner cortex, and core fractions and verify the cystine profiles derived from immunocytochemistry.

RESULTS. Qualitative and quantitative imaging approaches both showed that cystine labeling at the lens equator was most intense in the outer cortex, but diminished in the inner cortex before increasing again in the core. HPLC from the outer cortex, inner cortex, and core fractions confirmed high levels of cyst(e)ine in the core relative to the inner cortex. The bimodal distribution of cystine labeling in the outer cortex and core correlated well with the expression of the cystine–glutamate exchanger in these regions, but this was not the case in the inner cortex. A similar bimodal distribution of cystine labeling was observed in axial sections. However, in these sections a track of high-intensity cystine labeling was observed that was associated with the sutures indicating that the sutures act as a delivery pathway to the core.

CONCLUSIONS. These imaging approaches have revealed two distinct regions of cystine uptake in the outer cortex and core of the lens. Furthermore, this bimodal distribution of cystine has been shown to be the result of cystine delivery to the core via the sutures. This newly identified pathway opens an opportunity for the delivery of therapeutic antioxidants aimed at preventing age-related nuclear cataract. (Invest Ophthalmol Vis Sci. 2007;48:1253–1260) DOI:10.1167/iovs.06-0861

Old age nuclear cataract has been associated with oxidative damage.1 Glutathione (GSH) is the principal antioxidant in the lens, and its concentration has been shown to decrease with age, increasing the susceptibility of the lens to oxidative damage and the subsequent loss of transparency.2 GSH is a tripeptide synthesized from cysteine, glutamate, and glycine by the sequential actions of the enzymes γ-glutamylcysteine synthetase and glutathione synthetase.3–5 Cysteine has been identified as the rate-limiting substrate for GSH synthesis in the brain, kidney, liver, and pancreas.5–8 Cysteine is a sulfur-containing amino acid that is inherently unstable in free solution. However, cysteine, the dimeric oxidized form of cysteine, is more stable and able to travel freely through the blood where, after intracellular accumulation, it is then rapidly reduced to cysteine.9 In the eye, cysteine has been shown to be 50 times more abundant than cysteine in the aqueous humor of the guinea pig.10 Therefore, although the direct uptake of radioactive cysteine has been demonstrated in cultured monkey lenses,11 and cysteine synthesis from methionine via the transulfuration pathway has been shown,12 the uptake of cystine from the aqueous humor may be a more relevant mechanism for the accumulation of cysteine in the lens.

We have reported that the rat lens expresses Xc-, an amino acid transport system capable of mediating cystine uptake in a variety of tissues.13 Xc- is a heterodimer composed of a heavy chain (4F2hc) and a light chain (xCT).13 Cystine uptake is mediated by xCT and involves the exchange of extracellular cystine for intracellular glutamate. This exchange system relies on the maintenance of a high intracellular glutamate concentration, and, in other tissues, this is mediated by members of the XAG amino acid transport family.15,16 The XAG transporters are a multigene family of Na+-dependent amino acid transporters, which include the excitatory amino acid transporters (EAAT1 to -5) and the alanine serine cysteine transporters (ASCT1 and -2).17 We showed that xCT is expressed in the lens, but in combination with different members of the XAG family. In the outer cortex, xCT expression has been shown to overlap with EAAT4/5 expression, whereas in the core, xCT expression colocalizes with ASCT2 expression.13,18 We hypothesized that the observed switch in glutamate uptake mechanisms from EAAT4/5 to ASCT2 reflects the ability of ASCT2 to accumulate glutamate preferentially at low intracellular pH19—a finding consistent with the known acidic environment in the lens core.20 Thus, all fiber cells appear to contain the appropriate transport mechanisms for cystine–glutamate exchange and the recycling of glutamate to maintain the glutamate concentration gradient.

The question now arises as to whether the transporters in the different regions of the lens are actually functional. HPLC10,21 and autoradiography11 have been used to measure cyst(e)ine uptake. Unfortunately, these techniques do not provide the resolution necessary to make a direct correlation between cyst(e)ine uptake and the regional differences in Xc- expression we have observed. Homogenization of lens tissue for HPLC and autoradiography can provide an average cyst(e)ine concentration in a particular region, may not distinguish between intracellular and extracellular localization, and may not detect the cyst(e)ine gradients generated by specific patterns of Xc- expression.

In this article, we report on the development of an imaging approach to determine cyst(e)ine distributions in the rat lens at...
subcellular resolution. This approach utilizes high-quality histologic sections labeled with antibodies designed to detect free cystine.\textsuperscript{22} Regional differences in cystine distribution were revealed, which were correlated with the expression patterns of Xc- in different regions of the lens. The results suggest that uptake of cystine in the lens cortex and core occurs via two different pathways.

**Materials and Methods**

**Reagents**

Phosphate-buffered saline (PBS) was prepared from PBS tablets (Sigma-Aldrich, St. Louis, MO). The anti-rabbit cystine antibody was a generous gift from Robert Marc (John Moran Eye Center, University of Utah Health Sciences Center, Salt Lake City).\textsuperscript{22} The anti-rabbit amino terminal tail-specific xCT antibody was purchased from Transgenic, Inc. (Kumamoto, Japan). The goat anti-rabbit Alexa 488 secondary antibody and the membrane marker wheat germ agglutinin conjugated to tetramethyl rhodamine isothiocyanate (WGA-TRITC) were both obtained from Invitrogen-Molecular Probes (Eugene, OR). Unless otherwise stated, all other chemicals were obtained from Sigma-Aldrich.

**Animals**

All animals were treated according to the ARVO Statement for the Use of Ophthalmic and Vision Research and the University of Auckland Animal Ethics Committee. Twenty-one-day-old Wistar rats were killed by CO\textsubscript{2} asphyxiation. The eyes were removed and the lenses immediately extracted from the globe and placed in PBS.

**Immunocytochemistry**

Whole lenses were fixed (either in 0.75% paraformaldehyde and 0.01% glutaraldehyde or 0.75% paraformaldehyde alone), cryoprotected, and cryosectioned according to standard protocols developed in our laboratory.\textsuperscript{23} Sections cut in either an equatorial or axial orientation were washed three times in PBS and incubated in blocking solution (5% bovine serum albumin and 3% normal goat serum) for 1 hour to reduce nonspecific labeling. The sections were then labeled with the cystine antibody (1:400) or the xCT antibody (1:20) diluted in blocking solution to reduce nonspecific labeling. The sections were then labeled with the cystine antibody (green) and the membrane marker WGA (red). Strong cystine labeling was observed in the superficial layers of nucleated fiber cells (Fig. 1B). Strong cystine labeling was observed in the superficial layers of nucleated fiber cells. The sections omitted the primary antibody showed only WGA membrane labeling. A cystine intensity profile was then generated using Image J (available at www.imagej.nih.gov/ij or at http://rsb.info.nih.gov/ij/; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) and Origin ver. 5.0 (OriginLab, Northampton, MA).

**Quantitative Image Analysis of Cystine Labeling**

A series of image stacks from a z-series located at the periphery of the lens equator were captured.\textsuperscript{25} The brightest pixel values at every position in the x- and y-axis for each stack were projected onto one image to generate an extended maximum projection image to capture the cystine labeling fully. A series of overlapping image stacks were acquired along the equatorial radius of the lens extending from the periphery through to the lens core, resulting in a montage of cystine labeling. A cystine intensity profile was then generated using Image J (available at www.nimh.nih.gov/ij or at http://rsb.info.nih.gov/ij/; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) and Origin ver. 5.0 (OriginLab, Northampton, MA).

**High Performance Liquid Chromatography**

Whole lenses were dissected into three fractions: outer cortex, inner cortex, and core. Lenses were decapsulated and the adherent epithelial cells discarded. The superficial layers of nucleated fiber cells were peeled away and pooled as the outer cortex fraction. The inner cortical fiber cells were then removed to reveal a hard mass that corresponded to the core of the lens. All three lens fractions were homogenized in 5 mM HCl, 5 mM EDTA, and 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid. The homogenates were centrifuged at 12,000 rpm for 20 minutes and the supernatant retained, precipitated with the reducing agent Tris 2-carboxymethyl phosphine (100 mg/mL), and mixed with 10% trichloroacetic acid. The samples were then derivatized in 0.1% 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulfonate, 0.125 M boric acid, 4 mM EDTA, and 1.55 M NaOH and analyzed by reversed-phase HPLC (Alliance 2690; BAE Systems-AlphaTech, Burlington, MA). Standard curves and separations were performed using a C-18 column (3 μm C-18, 250 × 4.6 mm; Luna: Phenomenex, Torrance, CA) in gradient mode, at a flow rate of 800 μL/min. Absorbance was measured by a fluorescence detector operating at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. To determine the amount of cystine in each lens fraction, we weighed each lens fraction expressed the result as a percentage of whole lens weight (outer cortex 59%, inner cortex 23%, and core 12% ± 6%). These percentages were consistent with those previously calculated for human lenses (Veltman et al. IOVS 1993;34:ARVO Abstract 758).

**Statistical Analysis**

Statistical analysis was performed with a Student’s t-test to compare the data points obtained for cysteine levels in the three different fractions of the lens for significance. \( P < 0.05 \) was considered significant.

**Results**

**Immunodetection and Visualization of Cystine in Lens Sections**

Marc et al.\textsuperscript{22} have developed an extensive panel of antibodies that specifically recognize low-molecular-weight metabolites. These antibodies were raised by injecting a specific metabolite conjugated to bovine serum albumin by glutaraldehyde into rabbits. The antibodies specifically detected free metabolites fixed in place with glutaraldehyde.\textsuperscript{22} Antibodies produced by this design strategy have been successfully used to assay neurotransmitter levels in the retina.\textsuperscript{22,24,25} For our study, we have selected an antibody designed to detect free cystine to determine whether this immunohistochemical approach can be used in the lens.

Because the cystine antibody should only detect free cystine in fixed place with glutaraldehyde, a series of control experiments were developed to assess the antibody’s utility to monitor cystine levels in the lens (Fig. 1). Equatorial cryosections obtained from lenses fixed either in paraformaldehyde plus 0.01% glutaraldehyde (Fig. 1A) or paraformaldehyde only (Fig. 1B) were double labeled with the cystine antibody (green) and the membrane marker WGA (red). Strong cystine labeling was observed only in the presence of glutaraldehyde, indicating that the antibody only detects free cystine fixed with glutaraldehyde. Since the glutaraldehyde fixative exhibits some autofluorescence, an additional control was performed to ensure that the signal detected originated solely from cystine antibody labeling. Sections labeled from lenses fixed in both paraformaldehyde plus glutaraldehyde in which the primary cystine antibody was omitted showed only WGA membrane labeling (Fig. 1C) indicating autofluorescence from glutaraldehyde to be negligible.

Having established our controls in the cortex of the lens, we then extended our investigation of cystine labeling to other regions of the lens (Fig. 2). Representative images of free cysteine distribution were taken from four distinct regions of the lens (Fig. 2A). Cystine was detectable throughout the whole lens, except for the epithelial monolayer (Fig. 2B).
highest levels of cystine were observed in the outer cortex, a region that contains nucleated fiber cells (Figs. 2B, 2C). In this region (0 to 400 μm in from the capsule) cystine was primarily localized within the cytoplasm of the fiber cells. In the inner cortex (~400–800 μm in from the capsule), cystine was greatly reduced and appeared as punctuate cytoplasmic labeling (Fig. 2D). In the compacted core of the lens (~800–1200 μm in from the capsule), cystine levels appeared to increase relative to those in the inner cortex (Fig. 2E). In this region, cystine levels appeared highest close to the membranes of fiber

**FIGURE 1.** Antibody detection of free cystine in the cortex of the rat lens. Equatorial cryosections double labeled with the membrane marker WGA (red) and an antibody that recognizes free cystine conjugated to glutaraldehyde (green). (A) In lenses fixed in paraformaldehyde plus 0.01% glutaraldehyde strong cystine labeling is observed in fiber cells, but not the epithelium. (B) Lenses fixed in paraformaldehyde only and double labeled with the cystine antibody and WGA showed no cystine labeling, indicating that the antibody detects only free cystine conjugated to glutaraldehyde. (C) Lenses fixed in paraformaldehyde plus glutaraldehyde, but labeled with WGA alone, showed no evidence of cystine labeling, indicating glutaraldehyde autofluorescence is negligible. Scale bar, 16 μm.

**FIGURE 2.** Correlation of the labeling patterns of cystine and the Xc- in different regions of the rat lens. Equatorial cryosections double labeled with either cystine or Xc- antibodies (green), and the membrane marker WGA (red). (A) Montage of extended confocal images of a lens section labeled with WGA showing the areas from which high-magnification images (B–I) were recorded. (B–E) Representative images of cystine labeling in the epithelium (B), outer cortex (C), inner cortex (D), and core (E) of the lens showed regional differences in the distribution of cystine. (F–I) Representative images of xCT antibody labeling in the epithelium (F), outer cortex (G), inner cortex (H), and core (I) of the lens showed regional differences in the localization of the Xc.
cells. Thus, levels of cystine in the lens exhibit regional variations in signal intensity and subcellular location.

**Comparison of Free Cystine Levels and Cystine Transporter Localization**

In a previous study, we mapped the distribution of the cystine-glutamate exchanger (Xc-) using an antibody directed against the light chain (xCT) of this dimeric transport protein. This allows us now to compare the distribution of free cystine with the expression pattern of Xc-, an exchanger known to mediate cystine uptake. Like cystine, Xc- was expressed throughout the lens and similarly varied between lens regions. While Xc- was significantly expressed in the epithelium, only negligible levels of cystine were detected. This apparent inconsistency can be explained by the primarily cytoplasmic location of Xc- in the lens epithelium (Figs. 2B, 2F). In marked contrast to the epithelium, the adjacent fiber cells contained high levels of cystine (Fig. 2B). This striking step change in labeling intensity suggests that cystine does not diffuse via epithelial-fiber gap junctions, a finding consistent with previous reports of limited diffusion for small molecules between the two cell types. It seems more likely that the cortical fiber cells take up cystine themselves. It is puzzling therefore that Xc- is predominantly cytoplasmic, at least in the very outermost fiber cell layers (Fig. 2F). However, in slightly deeper-lying fiber cells (~40 cell layers in from the capsule), Xc- became more membranous in a region associated with high cystine uptake (Figs. 2C, 2G). Thus, it is theoretically possible that these deeper cells are supplying the more peripheral fiber cells with cystine via fiber-fiber gap junctions. In the inner cortex, the majority of Xc-labeling is associated with the broad sides of fiber cells, yet cystine levels are sharply reduced in this region (Figs. 2D, 2H). In the core region, Xc- is more uniformly spread in the fiber cell membranes, and cystine levels appear higher, albeit concentrated near the cell membranes (Figs. 2E, 2I). In these qualitative comparisons, it is difficult to assess the relative intensity levels of cystine. To achieve this, a more quantitative approach was developed to measure the levels of free cystine throughout the lens.

**Quantitative Mapping of Cystine Distribution from Labeled Sections**

It is technically challenging to quantify antibody labeling patterns accurately over large distances. Only very flat sections were used to minimize variations in signal intensity caused by artifactual changes in section thickness. Seamless image montages were assembled that captured the maximum signal intensity at every given depth within the section and also distance across the section. Since the signal intensity varied with distance into the lens, different photomultiplier tube (PMT) voltage settings were used in the collection of image stacks, to ensure that all signals from the cystine antibody were captured within an appropriate dynamic range (Fig. 3A). A calibration curve depicting pixel value as a function of voltage was constructed (Fig. 3C) and used to adjust individual image intensities for the changes in voltage, thereby producing a relatively smooth transition between image tiles.

**From the resultant image montage (Fig. 4A), an intensity profile was extracted to map the average signal intensity along the radius (~1200 μm) of an equatorial section. A representative intensity profile collected from a single lens section illustrates how the cystine labeling intensity varies from the lens periphery (excluding the epithelium) to a distance ~1600 μm into the lens (Fig. 4B).**
values normalized against the highest pixel value in each profile. For each profile, the distance into the center of the lens (r) was measured enabling the scale of relative distance (r/a) to be calculated for each lens. These normalized signal-intensity profiles could then be averaged and plotted against r/a (Fig. 4C) and confirmed that cysteine levels were consistently and significantly elevated in the outer cortex and the core relative to the inner cortex.

**Quantitation of Cysteine Levels across the Lens by HPLC**

We compared our immunocytochemical results to a more traditional biochemical approach for quantifying cyst(e)ine levels. Whole lenses were dissected into outer cortex, inner cortex, and core fractions and processed for HPLC. The inclusion of a reducing agent in the sample buffer resulted in the reduction of cystine to cysteine. Thus, this biochemical approach detects the average level of free cyst(e)ine in each fraction. Cyst(e)ine levels ranged between ~0.16 and 0.39 micromoles/g and were significantly higher in the core relative to both the outer and inner cortex (Fig. 4D). A similar elevation of cysteine in the core has been reported by others (Veltman et al. **IOVS** 1993;34:ARVO Abstract 758). The increase of cyst(e)ine in the core relative to the inner cortex detected by HPLC is consistent with our immunocytochemical observations. In contrast, the localized high levels of cystine in the outer cortex detected by immunocytochemistry were not evident by HPLC. Since the dissection and homogenization of each fraction destroys the spatial gradients of cysteine distribution, HPLC can only yield the average concentration of cyst(e)ine within a particular fraction. This averaging is particularly marked in the outer cortex, where the cysteine distribution is highly localized to the most peripheral cells. Thus, we expect that the average concentration of cyst(e)ine measured by HPLC underestimates the local peak concentration of cyst(e)ine in this fraction. Therefore, we attribute the discrepancy observed between the two methods in the outer cortex to the inability of the low-resolution biochemical technique to detect the localized cyst(e)ine gradient. Despite these uncertainties in the outer cortex, both approaches showed that cystine concentrations in the core were elevated relative to the inner cortex. Such a concentration gradient is difficult to explain by passive diffusion alone. Since the bimodal cystine intensity profiles were obtained using equatorial sections, we reasoned that it was possible that the elevated levels of cystine in the core were due to the preferential delivery of cysteine to the core via the sutures.

**Mapping a Sutural Pathway of Cysteine Delivery to the Lens Core**

Lens sutures are formed when elongating fiber cells meet at the lens poles. This end-to-end docking of fiber differs considerably between species producing sutures of varying complexity. The rat has a Y-shaped suture system of intermediate complexity. To investigate whether cysteine delivery to the lens core could be mediated by the lens sutures, we compared our immunocytochemical results to a more traditional biochemical approach for quantifying cyst(e)ine levels. Whole lenses were dissected into outer cortex, inner cortex, and core fractions and processed for HPLC. The inclusion of a reducing agent in the sample buffer resulted in the reduction of cystine to cysteine. Thus, this biochemical approach detects the average level of free cyst(e)ine in each fraction. Cyst(e)ine levels ranged between ~0.16 and 0.39 micromoles/g and were significantly higher in the core relative to both the outer and inner cortex (Fig. 4D). A similar elevation of cysteine in the core has been reported by others (Veltman et al. **IOVS** 1993;34:ARVO Abstract 758). The increase of cyst(e)ine in the core relative to the inner cortex detected by HPLC is consistent with our immunocytochemical observations. In contrast, the localized high levels of cystine in the outer cortex detected by immunocytochemistry were not evident by HPLC. Since the dissection and homogenization of each fraction destroys the spatial gradients of cysteine distribution, HPLC can only yield the average concentration of cyst(e)ine within a particular fraction. This averaging is particularly marked in the outer cortex, where the cysteine distribution is highly localized to the most peripheral cells. Thus, we expect that the average concentration of cyst(e)ine measured by HPLC underestimates the local peak concentration of cyst(e)ine in this fraction. Therefore, we attribute the discrepancy observed between the two methods in the outer cortex to the inability of the low-resolution biochemical technique to detect the localized cyst(e)ine gradient. Despite these uncertainties in the outer cortex, both approaches showed that cystine concentrations in the core were elevated relative to the inner cortex. Such a concentration gradient is difficult to explain by passive diffusion alone. Since the bimodal cystine intensity profiles were obtained using equatorial sections, we reasoned that it was possible that the elevated levels of cystine in the core were due to the preferential delivery of cysteine to the core via the sutures.
mediates the uptake of cystine which is then rapidly reduced to cysteine, an essential amino acid required for the production of GSH and other proteins. Because the extracellular space in the lens is ~<1% of the total lens volume, our measured intensity profiles for cystine effectively represent the intracellular accumulation of cystine. A schematic model summarizing the relationship between extracellular cystine, the subcellular location of Xc- and intracellular accumulation of cystine is shown in Figure 6A. In the epithelium, intracellular cystine levels were low, consistent with the apparent absence of Xc- in the plasma membrane. Our results are consistent with radiolabeled cystine- and cysteine uptake studies performed in epithelial cells, which showed low rates of uptake of both amino acids and minimal incorporation into GSH. In contrast, cystine levels in surface fiber cells were extremely high by immunodetection, suggesting that the two cell types exist as two separate compartments. This view is consistent with several

Distinction

Our adoption of an immunocytochemical approach enabled us to correlate free cystine levels more accurately to lens morphology and the localization of Xc-, a cystine-glutamate exchanger. Cystine levels were low in the epithelium and initially high in the outer cortex, but declined to a minimum in the inner cortex, before rising again in the core. The elevation of cystine in the core was confirmed by HPLC, both in the present study and in previous studies performed by Dickerson and Lou and Veltman and Lou (IOVS 1993;34:ARVO Abstract 758). This cystine profile cannot be explained by passive diffusion alone, and suggests that regional differences in the delivery and uptake of cystine exist in the rat lens.

Cystine is inherently more stable than its reduced form, cysteine, and as such is the form in which this amino acid is delivered to cells. In a variety of cell types, Xc- specifically

Figure 5. An alternative delivery pathway for cystine in the lens core. Axial sections were double labeled with the membrane marker WGA (red) and the cystine antibody (green). (A) Labeling of the membranes, clearly showed a suture line (arrows) that extended from the anterior pole to the core of the lens. (B) Labeling with the cystine antibody revealed cystine to be high in the outer cortex and low in the inner cortex, before increasing again in the core. (C) Merged image of (A) and (B) shows sites of high-intensity cystine labeling to colocalize with the sutures. Representative high-power images of cystine and membrane labeling in the inner cortex (D) and core (E) of the lens show strong labeling of cystine along the suture line and the membranes of fiber cells radiating from the suture. Scale bars: (A–C) 100 μm; (D, E) 10 μm.

Figure 6. A working model to explain regional differences in cystine distribution. (A) A cellular model of cystine uptake in different areas of the lens. The intracellular accumulation of cystine is only detected in those regions such as the cortex and core where Xc- is expressed in the membrane and a sufficient concentration of cystine is present in the extracellular space. The lack of cystine accumulation in the epithelium is due to the absence of Xc- in the membrane, whereas its absence in the inner cortex is due to the insufficient delivery of cystine as a result of restriction of the extracellular space. The exception to this relationship appears to be the superficial fiber cells that lack membranous Xc- but exhibit high levels of cystine accumulation. It is proposed that a gap junction-mediated pathway links these superficial cells to deeper fiber cells that contain a functional Xc- uptake pathway. (B) Proposed model for cystine delivery in the rat lens. The bimodal cystine-intensity profile (black line) has been overlaid on a schematic of lens structure to illustrate the alternative delivery pathways for cystine. In the outer cortex, cystine delivery occurs either via direct diffusion from the surrounding aqueous humor (blue arrows) or via axial diffusion from the sutures (dashed red arrows). In the inner cortex, extracellular delivery of cystine is limited by a restriction of the extracellular space causing cystine levels to reach a minimum in this region. In the core, cystine delivery is mediated by the sutures (red arrows).
dye-transfer studies, which have demonstrated either an absence of epithelial-to-fiber cell communication, or only limited dye transfer between the two cell types in specific regions of the lens. The existence of a steep fiber to epithelial concentration gradient for cystine shows that fiber cells are capable of direct uptake of cystine and do not solely depend on the epithelium for nutrient uptake.

Like the epithelium, superficial fiber cells contained a cytoplasmic pool of Xc; however, in contrast to the epithelium these newly differentiated fiber cells exhibited high levels of cystine labeling (Fig. 6A). The cytoplasmic location of Xc- in these fiber cells indicates that these cells are incapable of direct uptake of cystine from the extracellular space. This suggests that superficial fiber cells are supplied with cystine via a gap junction-mediated pathway, which links them to fiber cells deeper in the cortex that express Xc- in their membranes. This idea is consistent with the highest level of intracellular cystine coinciding with the insertion of Xc- into the membrane of differentiating fiber cells. It also highlights the known differences in the permeability properties of epithelial-fiber and fiber-fiber cell gap junctions.

From this peak of cystine accumulation located some ~120 μm in from the capsule, intracellular cystine levels decline to a minimum level—a pattern expected of diffusion-limited delivery of cystine to deep-lying fiber cells in the outer cortex. Thus, although cells deeper in the outer cortex express Xc-, insufficient cystine is delivered to them, making cystine accumulation difficult to detect (Fig. 6A). In the inner cortex, the fall-off in intracellular cystine suddenly plateaus before eventually rising again in the core, a pattern inconsistent with passive diffusion. We can conceive of three possible scenarios that could account for the observed plateau in the cystine intensity profile: (1) an inability to deliver extracellular cystine to the inner cortex; (2) an inhibition of Xc- mediated cystine uptake; (3) or an increase in the rate of conversion of cystine to cysteine and its subsequent utilization for GSH synthesis. Of these three scenarios, the last two seem less probable than the first. Inhibition of Xc- in the inner cortex appears unlikely, as the exchanger would have to be reactivated in the older cell in the lens. Our HPLC results showed a similar reduction of the cystine concentration in the inner cortex relative to levels in the core (Fig. 4D). In addition, this analysis showed no elevation of GSH in the inner cortex (data not shown), indicating that the rapid conversion of cystine to cysteine and its incorporation into GSH is not a probable cause for the low levels of cystine detected in this region. In contrast, the scenario of impaired delivery to the inner cortex is supported by the observation that the insertion of the adhesion protein MP20 in the inner cortex restricts the extracellular diffusion of small molecules, thereby limiting the delivery of cystine to functional Xc- transporters located in this area. The high levels of cystine observed in the lens core suggest that a secondary extracellular delivery route exists that bypasses this zone of extracellular space restriction and directly supplies cystine to the lens core where it is accumulated by Xc-.

In axial sections taken through the lens poles, it is apparent that this alternative delivery route resides in the lens sutures (Fig. 6B). In the rat lens sutures are formed by the end-to-end contact of fibers primarily within hemispheres. In each growth shell, the end-to-end contact of fibers occurs along three ~60° latitudinal arc lengths oriented at 120° longitudinal to one another to form an upright anterior Y suture, and because of opposite end fiber curvature, an inverted posterior Y suture. Thus, in the rat lens six offset, radially defined triangular delivery pathways are created from the periphery to the core in these lenses. The extracellular delivery of cystine via the sutures is consistent with the view of lens transport encapsulated by the microcirculation system. This circulation system is proposed to be driven by a standing flow of ionic current that is directed inward at the poles and outward at the equator. Briefly, the working model is that current which is carried by Na+ and Cl− ions, enters at the poles and travels into the lens via the extracellular space between fiber cells. The ions cross the fiber cell membranes, and return toward the surface via an intracellular pathway mediated by gap junction channels. The model predicts that this circulating flux of ions is generated by spatial differences in ion transport properties. This ion flux drives a fluid flow that convects nutrients such as cystine into the lens via the extracellular space where they can be accumulated intracellularly by transporters like Xc-. In the cortex, the pattern of cystine accumulation and the membrane expression of Xc-suggest that the extracellular delivery of cystine is sufficient only to supply the outer fiber cells (Fig. 6B). However in the core, extracellular delivery of cystine via the suture supplies sufficient cystine for uptake by Xc- into central fiber cells. This accumulated intracellular cystine would then be free to diffuse from the center toward the inner cortex, producing the observed bimodal cystine intensity profile.

The significance of the bimodal cystine distribution to overall lens transparency may be explained by proposing different roles for cysteine in the outer cortex and core. In the outer cortex, the major role for cysteine is to act as a precursor amino acid for GSH synthesis. However, in the core, a region that lacks the capacity to synthesize GSH, cysteine is proposed to act as a low-molecular-weight antioxidant by maintaining the free sulfhydryl groups of proteins in their reduced state. By analogy to the GSH/GSSG cycle, we propose a similar antioxidant role for cysteine/cystine. After exposure to reactive oxygen species, cysteine is temporarily oxidized to reform cystine. NADH- and NADPH-induced reduction of cystine then regenerates cysteine. We envisage this cycle of cystine-cysteine conversion plays a critical role in maintenance of reductive redox balance in the lens core which protects against oxidative damage. Studies have shown that the lipid bilayer of fiber cell membranes is most susceptible to oxidative damage, which could explain our observed association of cysteine with the membrane in the core. The different roles played by cysteine in the cortex and core, both contribute to the antioxidant status of the lens and are consistent with the observation that cysteine prodrugs are effective antiglaucoma therapies.

In conclusion, we have detected elevated cystine levels in the lens core that correlate well with the presence of membrane bound Xc- and a delivery pathway via the sutures. Having validated this technique for cystine, this approach can now be expanded to map GSH metabolism pathways throughout the lens using commercially available metabolite specific antibodies. By fully characterizing GSH pathways in different areas of the lens, we hope to elucidate the molecular mechanisms responsible for antioxidant uptake and how antioxidant depletion initiates age-related cataracts.

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References


