Molecular Identification of P-Glycoprotein: A Role in Lens Circulation?

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PURPOSE. To determine whether P-glycoprotein is expressed in the rat lens and to assess what type of damage occurs when Pglycoprotein inhibitors are applied to organ-cultured lenses.

METHODS. An initial screening for the P-glycoprotein isoforms multidrug resistance (mdr)1a, mdr1b, and mdr2 was performed by RT-PCR on RNA extracted from rat lens fiber cells. Northern blot analysis was used to determine whether transcript levels detected by RT-PCR were significant. The presence of P-glycoprotein in the lens was confirmed by Western blot analysis and immunocytochemistry. Organ-cultured lenses, maintained in isotonic artificial aqueous humor, were exposed to various concentrations of the P-glycoprotein inhibitor tamoxifen. Lens opacification was assessed by dark-field microscopy, and the underlying cellular changes were visualized by confocal microscopy of lens sections, using a fluorescent membrane marker. Initial cellular damage was assessed after a 6-hour exposure to 100 μM tamoxifen. Other P-glycoprotein inhibitors, verapamil, and 1,9-dideoxyforskolin (DDFK) were assessed, and the damage phenotypes were compared with those seen for tamoxifen.

RESULTS. Transcript for all three P-glycoprotein isoforms was detected with RT-PCR, but only mdr1a and mdr2 could be detected by Northern blot analysis. P-glycoprotein was localized in the plasma membrane of lens epithelial and fiber cells. Treatment of organ-cultured lenses with increasing doses of the P-glycoprotein inhibitor tamoxifen for 18 hours showed that two distinct damage phenotypes were evident. At a dose of 20 μM tamoxifen, tissue damage was found in a discrete zone that initially started approximately 100 μm from the capsule, whereas at higher doses (60–100 μM tamoxifen), extensive vesiculation of fiber cell membranes occurred throughout the entire lens cortex. Decreasing tamoxifen (100 μM) exposure to 6 hours showed that the inner zone of damage was caused by the dilation of extracellular space between fiber cells. The extracellular space dilation and fiber cell vesiculation could be reproduced by varying the concentrations of the P-glycoprotein inhibitor tamoxifen. Lens opacification was assessed by dark-field microscopy, and the underlying cellular changes were visualized by confocal microscopy of lens sections, using a fluorescent membrane marker. Initial cellular damage was assessed after a 6-hour exposure to 100 μM tamoxifen. Other P-glycoprotein inhibitors, verapamil, and 1,9-dideoxyforskolin (DDFK) were assessed, and the damage phenotypes were compared with those seen for tamoxifen.

CONCLUSIONS. The P-glycoproteins mdr1a and mdr2 are expressed in the lens and appear to be functional. The initial cellular damage phenotype of extracellular space dilations caused by the P-glycoprotein inhibitors was identical with that caused by chloride channel inhibitors, indicating that P-glycoprotein may play a role in regulating cell volume in the lens. Whether the secondary damage phenotype of fiber cell vesiculation, induced by high doses of P-glycoprotein inhibitors, was due to the inhibition of additional regulatory activities of P-glycoprotein or to nonspecific effects of the drugs remains to be determined. However, regardless of the precise mode of action, these results indicate that P-glycoprotein should be considered in the regulatory mechanisms associated with the control of lens volume and in the initiation of osmotic cataract. (Invest Ophtalmol Vis Sci. 2002;43:3008–3015)

P-glycoproteins are highly conserved multifunctional membrane proteins that act as adenosine triphosphate (ATP)-dependent efflux pumps that may confer multidrug resistance or act as modulators of chloride channels that regulate cell volume.1 Although P-glycoprotein has not yet been identified molecularly in the lens, several observations suggest that it is present and may modulate chloride channel activities. Both, tamoxifen, an inhibitor of P-glycoprotein, and an antibody specific for P-glycoprotein have been shown to block the chloride channel in lens fiber cell membranes.2,3 Furthermore, osmotic cataract develops in organ-cultured lenses exposed to tamoxifen.2 These observations suggest that chloride channels are expressed in the lens, and the findings are of interest because they support the existence of a circulating flux of chloride ions in the resting lens.4

The existence of a circulating flux of chloride ions in the lens was first proposed by Mathias et al.5 Such a flux is consistent with the strengthening view that the lens maintains nutrient homeostasis and regulates cell volumes by using an internal microcirculation system.5,6 The circulation model contends that an extracellular fluid flux converges nutrients deep into the lens cortex where the fiber cells can take up the nutrients along with sodium ions, chloride ions, and water. Metabolic wastes, ions, and water are drawn back to the lens periphery through a cytoplasmic route mediated by gap junction channels. This circulation system is driven by sodium pumps and potassium channels, which are concentrated at the lens periphery. The spatial distribution of ion channels and pumps creates a radial gradient of membrane potentials that declines toward the center of the lens. Because chloride distributes itself across the fiber cell membrane in accordance with its electrical chemical equilibrium potential (EC1), the direction of chloride ion movements vary as a function of radial depth in the lens.7 Near the periphery, the membrane potential is more negative than the E Cl, and favors the efflux of chloride ions, and therefore water, from these cells. Deeper in the lens, the membrane potential is less negative than the E Cl and favors the influx of chloride ions and water into the fiber cells.

Evidence supporting the existence of such a circulating chloride flux in the lens is based on the morphologic examination of the effects of the chloride channel inhibitor 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB). Exposure of organ-cultured rat lenses to NPPB results in significant tissue damage.6,7 Characteristically, the lenses take up water, which

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accompanies between the fiber cells in a discrete zone of the inner cortex and also inside the fiber cells that are located closer to the lens periphery. This occurs because NPPB blocks chloride influx in the deeper lying fiber cells, causing an accumulation of extracellular fluid that produces the observed localized zone of extracellular space dilations. In the more peripheral fiber cells NPPB prevents chloride efflux, producing an intracellular accumulation of fluid and subsequent cell swelling.

Pglycoprotein’s role as a modulator of chloride channel activity indicates that it could act to regulate this circulating flux of chloride ions and therefore indirectly regulate lens hydration and transparency. However, before such a functional relationship can be credibly investigated, several uncertainties must be resolved. Presently, there appears to be no published evidence at the molecular level that demonstrates that P-glycoprotein is indeed expressed in the lens. Furthermore, it is not known whether blocking chloride channels indirectly with inhibitors of Pglycoprotein causes the same kind of tissue damage observed when chloride channels are directly blocked. In our study, we used the rat lens model to investigate these uncertainties. We found that transcripts for several P-glycoprotein isoforms were expressed in the lens, and that Pglycoproteins were present in the plasma membranes of both lens epithelial and fiber cells. We further showed that although tamoxifen and other Pglycoprotein inhibitors caused widespread vesiculation of the fiber cell membranes and opacification of organ-cultured lenses, histologic changes in the initial stages of this process resembled those observed after treatment with NPPB. This suggests that Pglycoprotein may be involved in regulating the circulating chloride fluxes, which have been implicated in the regulation of lens volume.

**Materials and Methods**

**Chemicals**

NPPB was obtained from Research Biochemicals, Inc. (Natick, MA). Other chemicals including tamoxifen, verapamil, DDFK, and fluorescein-conjugated wheat germ agglutinin (FITC-conjugated WGA, *Triticum vulgaris*) were obtained from Sigma Chemical Co. (St. Louis, MO). The antibody goat anti-multidrug resistance (mdr) protein (C129) was obtained from Research Diagnostics (Flanders, NJ).

**RT-PCR Screening for P-Glycoprotein Isoforms**

All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Three- to 4-week-old Wistar rats were killed by CO2 asphyxiation and the eyes extracted. Whole lenses were extracted from the eyes in sterile dimethyl dicarboxylate (DMDC)-treated phosphate-buffered saline (PBS: 2.7 mM KCl, 10 mM phosphate buffer, 137 mM NaCl [pH 7.4]). Lenses for RNA extraction were rolled on sterile filter paper to remove any adherent tissues, decapsulated to remove adherent epithelial cells, and immediately placed in a tissue storage reagent (RNAlater; Ambion, Austin, TX) accurately placed in a tissue storage reagent (RNAlater; Ambion, Austin, TX) and subsequently sequenced by the dideoxy chain termination technique and a DNA sequencer (373A stretch DNA sequencer, Applied Biosystems, Foster City, CA) on 5.25% polyacrylamide gels.

**Northern Blot Analysis**

Total RNA (5-10 μg) was loaded on 1% agarose denaturing gels (1% agarose (Ultrapure; Roche Molecular Biochemicals), 1× 5-(N-morpholino)propanesulfonic acid (MOPS), and 2.2 M formamide). After electrophoresis at 5 V/cm for approximately 4 hours, total RNA was transferred onto a nylon membrane (Roche Molecular Biochemicals) overnight. The transferred denatured RNA was fixed onto the nylon membrane with a 3-minute exposure to UV light. The membrane was incubated in 6 mL of solution (DIG Easy Hyb; Roche Molecular Biochemicals) for 30 minutes before hybridization. Sequenced confirmed gene-specific DNA template (1 μg) was labeled with digoxigenin-11-dUTP in 20-μL reactions, using the DNA labeling kit (DIG; Roche Molecular Biochemicals). Double-stranded DIG labeled probes (10-20 ng/mL in DIG Easy Hyb) were heat denatured at 99°C for 10 minutes, and hybridization was performed for approximately 3 to 4 hours at room temperature. The unbound probe was removed by washing the nylon membrane with ten 2× SSC and 0.1% SDS washes with gentle agitation at room temperature. A further two washes (0.5× SSC and 0.1%SDS) were performed at the hybridization temperature for 10 minutes to remove nonspecific binding of the probe. The nylon membrane was washed in buffer A (100 mM maleic acid, 150 mM NaCl [pH 7.5]) at room temperature for 1 minute, and nonspecific antibody binding was blocked with a 30-minute incubation in blocking buffer (1% nonfat milk powder in buffer A). After labeling with the anti-DIG antibody diluted 1:20,000 (Roche Molecular Biochemicals), DNA-RNA hybrids were detected with chemiluminescence (CDP-star; Roche Molecular Biochemicals) exposed on autoradiographic film (Hyperfilm, ECL; Amersham, Little Chalfont, UK). Film was developed in an automatic processor (Curix 60, AGFA; Nunading, Victoria, Australia).

**Table 1.** P-Glycoprotein Primer Sets

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<th>GenBank Accession No.</th>
<th>Oligonucleotide</th>
<th>Expected PCR Product Size (b)</th>
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<td>Sense (20 b, position 46) TTACGGAGAAATCGGAAAG</td>
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<tr>
<td>Antisense (20 b, position 1090) AGAGAGAAGGCGCCGAAGAA</td>
<td>1131</td>
<td></td>
</tr>
<tr>
<td>Pgp3 (mdr2) L15079</td>
<td>Sense (21 b, position 1180) AGCCACAGACATCAAGAA</td>
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<td>Antisense (21 b, position 2288) CCCAGAAGGCGCCGAAGAC</td>
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Detection of P-Glycoprotein by Western Blot Analysis and Immunocytochemistry

Crude membrane proteins were isolated from 10 adult rat lenses or from 1 adult rat brain. Tissue was homogenized in 10 mL of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg/mL leupeptin. The homogenate was centrifuged at 900g for 10 minutes at 4°C, and the resultant supernatant was centrifuged at 24,000 rpm (SW 40Ti; Beckman Instruments, Fullerton, CA) for 75 minutes at 4°C. The pellet was solubilized by incubation for 1 hour in 500 μL buffer (100 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.5% TritonX-100, 0.5% sodium deoxycholate, and 1 mM PMSF). This homogenate was centrifuged at 14,000g for 10 minutes at 4°C, and the supernatant, containing the solubilized proteins, was collected in a separate tube. Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a nitrocellulose membrane by electrophoresis for 90 minutes at 170 mA. Transfer of proteins was confirmed by staining the nitrocellulose membrane for 4 minutes with Ponceau stain (1% Ponceau, 1% acetic acid in 100 mL filtered H2O (MilliQ; Millipore, Bedford, MA)). Membranes were incubated overnight at room temperature in a blocking solution (1% BSA, 0.1% Tween20, 2 mM Tris-HCl, 140 mM NaCl [pH 7.6]) and subsequently incubated for 2 hours with goat anti-mdr protein antibody diluted 1:1000 (0.2 μg/mL in 1%BSA, Tris buffered saline [TBS]). Membranes were then exposed to biotinylated anti-goat IgG secondary antibody (Amersham) diluted 1:1000 for 1 hour, followed by streptavidin-horseradish peroxidase (HRP; Amersham) diluted 1:1000. After each incubation, the membranes were rinsed three times with water and washed three times for 15 minutes in TBS. The presence of P-glycoprotein was detected by enhanced chemiluminescence and exposed on autoradiographic film (Hyperfilm, ECL; Amersham).

For immunocytochemistry experiments, whole lenses were fixed for 16 hours in 2% paraformaldehyde in PBS. Wholomount capsules, with adherent epithelial cells, were fixed for 15 minutes. Lenses were briefly rinsed in PBS, incubated in 30% sucrose for 6 hours before incubating in optimal cutting temperature (OCT; Sakura Finetek, Tokyo, Japan) overnight. Whole lenses were mounted in OCT and frozen, and sections were cut at 16 μm. After three 5-minute washes in PBS, the lens sections or epithelial wholemounts were incubated in goat anti-mdr protein antibody diluted 1:50 (4 μg/mL in PBS) for 2 hours at room temperature. A control section, with goat anti-mdr protein antibody preincubated with peptide was also prepared. After three 5-minute washes in PBS, the sections were incubated with a secondary antibody, anti-goat IgG conjugated to red dye (Rhodamine Red; Molecular Probes, Eugene, OR) diluted 1:100. Lenses were washed with three 15-minute washes in 1× PBS before the sections were mounted onto slides with antifading reagent (Fluoroguard; Bio-Rad, Richmond, CA). Sections were viewed with a confocal laser scanning microscope (TCS 4D, Leica Lasertechnik, Heidelberg, Germany) fitted with an argon-krypton mixed gas laser. The software accompanying the microscope was used to record confocal microscopy images (SCAware; Leica).

Morphologic Analysis of Inhibitor Effects on Lens Architecture

Lenses were carefully removed from the globe and placed immediately in artificial aqueous humor (AAH: 125 mM NaCl, 4.5 mM KCl, 10 mM NaHCO3, 2 mM CaCl2, 0.5 mM MgCl2, 5 mM glucose, 20 mM sucrose, 1% penicillin-streptomycin, and 10 mM HEPES [pH 7.4], osmolality 300 mmol/kg) at 37°C for 1 hour and screened visually for damage. Lenses in which opacities developed during this incubation were discarded. Lenses were either left in AAH or transferred to AAH containing NPPB (100 μM), tamoxifen (2, 20, 60, or 100 μM), DDFK (100 μM), or verapamil (100 μM) for 6 or 18 hours at 37°C before being prepared for histologic analysis. NPPB and DDFK were dissolved in dimethyl sulfoxide (DMSO; 0.1% vol/vol), and tamoxifen and verapamil were dissolved in methanol (0.2% vol/vol). Neither DMSO nor methanol, when added in the absence of drugs, had any effect on lens tissue architecture at the concentrations used. After treatment with inhibitors, lenses were fixed in 25% Karnovsky’s solution (50 mM Na cacodylate, 1% paraformaldehyde, 1.25% glutaraldehyde) in PBS (pH 7.4; osmolality 300 mmol/kg) for 4 hours at room temperature. Fixed lenses were super-glued to the plate of a vibratome (Vibratome 1000; Technical Products International, Inc., St. Louis, MO). Equatorial or axial 170-μm thick sections were cut. Sections were incubated in FITC-conjugated WGA (1 μg/mL in PBS) overnight in the dark at room temperature. Sections were then given four 10-minute washes in PBS. Labeled sections were mounted in antifade medium (Citifluor; Agar Scientific, Stansted, UK) to reduce fading and were examined by confocal microscopy.

RESULTS

Molecular Identification of P-Glycoprotein in the Rat Lens

Three rodent isoforms of P-glycoproteins are known: mdr1a and mdr1b, which are related to each other by gene duplication and mdr2.8-11 Whereas mdr1a and mdr1b both function as ATP-dependent transporters and can confer multidrug resistance, mdr2 appears to be involved in phospholipid transport.11,12 Of particular interest in the context of this report is the additional ability of mdr1a to modulate a volume-sensitive chloride conductance associated with cell volume regulation in a variety of cells.13 We have designed PCR primers sets capable of amplifying specifically each isoform (Table 1). Conditions for PCR were optimized for cDNA obtained from rat kidney, a tissue known to express all three isoforms.13,14 When these conditions were applied to cDNA from rat lens fiber cells, PCR products of appropriate sizes were amplified for mdr1a, mdr1b, and mdr2 (Fig. 1A). The identity of these PCR products was confirmed by sequencing (data not shown).

The RT-PCR procedure is capable of detecting minute levels of transcript. To determine whether P-glycoprotein transcripts are expressed at significant levels in the lens, Northern blot analysis was performed using lens fiber and epithelial total RNA (Fig. 1B). It is evident from the stained gel (Fig. 1B, I) that the loading of epithelial RNA was less than that from the fiber cells. Amounts of epithelial RNA were limited by the relatively small percentage of these cells in the lens and our reluctance to kill a larger number of animals. Probe specificity and hybridization conditions were confirmed, with the kidney serving as a control tissue. Transcript for mdr1a probe was detected in lens fiber cells (Fig. 1B, II), whereas mdr2 was detected in both epithelial and fiber cells (Fig. 1B, IV). Transcript levels for mdr2 were more pronounced in the epithelium, a result made more significant because there was less epithelial than fiber cell RNA loaded on the gel. This indicates that the epithelium expresses most mdr2 found in the lens. Transcript for mdr1b was not detected by Northern blot analysis (Fig. 1B, III), suggesting that this isoform is not abundantly expressed in the lens.

To verify that P-glycoprotein was not only present at the transcript but also the protein level, Western blots were prepared from gels separating total membrane proteins of rat lens fiber cells. The commercially available antibody used for this purpose, C129, was specific to the carboxyl tail peptide sequence common to all three P-glycoprotein isoforms. A protein of the predicted size of approximately 170 kDa and another protein of 100 kDa, probably a proteolytic cleavage product, were detected in the lens and the control tissue, brain (Fig. 1C). Immunocytochemical labeling of lens sections with the same antibody localized P-glycoprotein to the plasma membrane of both epithelial (Fig. 2) and fiber cells (Fig. 5). Labeling was not detected in neither epithelial wholemounts (Fig. 2B) nor lens sections (data not shown) when incubated in the presence of P-glycoprotein antibody that was preabsorbed.
with the P-glycoprotein–specific peptide. An optical section taken through the middle of the wholemount showed staining throughout the epithelial cell membrane (Fig. 2A). To more fully localize P-glycoprotein distribution, a series of images
were taken through the wholemount. The reconstructed image shows that, although P-glycoprotein was distributed throughout the epithelial cell layer, it was predominantly localized to the apical membrane domain (Fig. 2C). Staining for P-glycoprotein was found in the fiber cells throughout the entire lens cortex (Figs. 3A, 3C). This labeling was punctate in appearance and distributed around the entire fiber cell profile (Fig. 3B, 3D). Taken together, our results indicate that the protein encoding for P-glycoprotein was expressed in both epithelial and fiber cells in amounts that could be detected in the rat lens by immunocytochemistry. However, because the commercial antibody used in this study recognizes all three isoforms of P-glycoprotein, a more definitive localization of the specific isoforms could not be performed.

**Pharmacologic Effect of P-Glycoprotein Inhibitors on Lens Architecture**

Cellular changes resulting from the inhibition of P-glycoprotein were assessed by incubating lenses in various concentrations of tamoxifen and examining them by dark-field microscopy. Lenses were also fixed and sectioned, and cell membranes were stained with FITC-conjugated WGA to facilitate the visualization of changes to tissue architecture. Exposure to 2 μM tamoxifen for 18 hours had no noticeable effect on tissue architecture or lens transparency, as the highly ordered structure of the fiber cells was perfectly evident (Fig. 4A). Exposure to 20 μM tamoxifen produced a lens equatorial opacification with occasional projections toward both poles (Fig. 4B, inset). The tissue damage responsible for this opacification was a highly localized zone of fiber cell disruption, 50 to 100 μm inward from the lens capsule (Fig. 4B). The extent of both tissue damage and opacification increased significantly after exposure to 60 μM tamoxifen (Fig. 4C, inset). Treatment with 100 μM tamoxifen resulted in the complete vesiculation of fiber cells throughout the lens cortex, with concomitant clouding of the entire lens (Fig. 4D, inset).

To characterize further the initial stages of tamoxifen-induced tissue damage, we shortened the treatment time to 6 hours. Sections were cut in both equatorial and axial planes to achieve a high definition of cellular changes (Fig. 5). After a 6-hour incubation in 100 μM tamoxifen, tissue disruptions were confined to a discrete zone approximately 100 μm inward from the capsule (Figs. 5A, 5C) and consisted of minor irregularities in the otherwise crystalline packing of fiber cells. At higher magnification, cystlike dilations of extracellular spaces were demonstrated (Figs. 5B, 5D). In contrast, an 18-hour incubation in 100 μM tamoxifen, caused extensive fiber cell disintegration in both equatorial and axial sections of the lens (Figs. 5E–H). The similarity between the damage phenotype in equatorial and axial sections indicates that the fiber cells were being broken up into relative homogeneous population of fiber cell membrane vesicles (Figs. 5F, 5H).

**Effects of Other P-Glycoprotein Inhibitors on Fiber Cell Morphology**

Although tamoxifen is a potent inhibitor of P-glycoprotein, the fact that there were two distinct time- and dose-dependent damage phenotypes indicates that tamoxifen could have additional effects on other proteins. To investigate this possibility, two other P-glycoprotein inhibitors verapamil and DDFK were applied to the lens (Fig. 6). Lenses exposed to 100 μM verapamil for 18 hours showed extracellular space dilations (Fig. 6A) similar to those that were observed after exposure to 100 μM tamoxifen for 6 hours (Fig. 5A). Exposure of lenses to 100 μM DDFK produced a mixture of tissue damage types with extracellular space dilations becoming obscured by the initiation of fiber cell vesiculation (Fig. 5B). This damage phenotype strongly resembled that which occurred after exposure of lenses to 60 μM tamoxifen for 18 hours (Fig. 4C). Increasing the dose of verapamil to 200 μM for 18 hours induced extensive fiber cells vesiculation (Fig. 6C), similar to that which occurred after an 18-hour exposure to 100 μM tamoxifen (Fig. 5E). Thus, a comparison of the tissue damage phenotypes for the three P-glycoprotein inhibitors suggests that tamoxifen is more potent than DDFK, which is more potent than verapamil.

**DISCUSSION**

Osmotic insults are believed to be an initiating factor in most cortical cataracts. This is because any insult to the lens that perturbs lens volume disrupts the regular packing of fiber cells and increases light scattering. Most prominently, inhibitors of membrane transport proteins associated with the lens circulation system cause osmotic tissue damage. Recently, it has been shown that inhibitors of chloride channels disrupt the circulating flux of chloride ions and water, causing a localized dilation of extracellular space deeper in the cortex and cell swelling nearer to the periphery. Our present report provides the first evidence that P-glycoprotein, known as a regulator of volume-sensitive chloride channels in other tissues, is actually expressed in the lens. Inhibitors of P-glycoprotein cause initial tissue damage that consists of extracellular space dilations, similar to those caused by blocking chloride channels. This strongly suggests that P-glycoprotein inhibitors block the circulating chloride fluxes predicted by the lens circulation model.

Transcripts of all three isoforms were detected by RT-PCR, but only mdr1a and mdr2 were detected by Northern blot analysis, indicating that these two isoforms are abundantly expressed in the lens. Of these two isoforms, mdr1a is the likely target for tamoxifen, because mdr1a is expressed in the fiber cells and has the ability to regulate chloride channels. The other isoform present in the lens, mdr2, is highly expressed in the epithelium and is thought to be a phospholipid transporter. Transgenic mice that overexpress the human homologue of mdr2 (MDR3) in the fiber cells have cataract. It
is thought that overexpressing the phospholipid transporter affects membrane structure and therefore the activity of membrane transport proteins. Such perturbation is likely to involve longer time scales than was applicable in our experiments, which were based entirely on short-term lens organ cultures. Hence, we suggest that mdr1a is the relevant isoform that is involved in the tamoxifen-induced changes documented in our present report.

The degree of tissue damage caused by tamoxifen was dose and time dependent. An 18-hour exposure to a physiologically more relevant concentration of 2 mM tamoxifen did not result in any visible signs of tissue perturbation. At a dose of 20 μM tamoxifen, a reproducible localized zone of tissue damage was observed that had a similar location to the damage induced by NPPB in a previous study conducted in our laboratory. In both cases, fiber cell damage was initiated by extracellular space dilations. Prolonged exposure to tamoxifen caused damage throughout the entire cortex, which was characterized as fiber cell vesiculation. This vesiculation damage phenotype did not occur in NPPB-treated lenses. Vesiculation of fiber cells is a phenomenon seen in many types of cortical cataracts, and in particular when fiber cells are isolated in medium containing calcium. This latter phenomenon has been attributed to membrane depolarization, consequential influx of calcium, and activation of calcium-dependent proteases. How can these phenomena be reconciled into a consistent inhibitory action for tamoxifen in the lens? We propose that, at short exposure times or at low doses, tamoxifen induces extracellular space dilation by blocking chloride flux into these deeper fiber cells. Whether tamoxifen acts through blocking chloride channels directly, or through inhibition of P-glycoprotein is difficult to determine from our experiments. However, it is now generally accepted that P-glycoprotein has no inherent chloride channel activity but rather modulates the activity of a chloride channel. This is supported by the observation that verapamil and DDFK, which have no effect on swelling-induced chloride conductances in a variety of cell types, both produced localized extracellular space dilations in the lens. Hence, it is likely that the action of tamoxifen at low doses is mediated by P-glycoprotein and not by the direct inhibition of a chloride channel.

At longer exposure times and higher doses, tamoxifen and the other P-glycoprotein inhibitors caused fiber cell vesiculation. This indicates that P-glycoprotein inhibitors have multiple

**FIGURE 5.** Tamoxifen induces two distinctive damage phenotypes. FITC-WGA–labeled sections from rat lenses incubated in AAH–100 μM tamoxifen for 6 hours (A–D) or 18 hours (E–H). Equatorial (A, B) and axial (C, D) sections show that exposure to 100 μM tamoxifen for 6 hours caused extracellular space dilations between fiber cells (B, arrows). Equatorial (E, F) and axial (G, H) sections show that exposure to 100 μM tamoxifen for 18 hours induced fiber cell vesiculation (H). High-power image was taken deeper in the lens to highlight the interface between normal fiber cells (upper right) and those cells that had vesiculated (E). (B, D, F) High-power images of sections shown in boxes in (A), (C), and (E), respectively. Cap, capsule; Ep, epithelium.
effects on the ability of the lens to regulate volume. In mouse neuroblastoma cells, tamoxifen has been shown to activate a volume-sensitive maxi-chloride channels by P-glycoprotein: phosphorylation has the final say.  

**References**


