Transcytotic Passage of Albumin through Lens Epithelial Cells

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Purpose. To characterize the transcytotic passage of albumin through lens epithelial cells.

Methods. N/N 1003A rabbit lens epithelial cells were grown to a confluent monolayer on porous filter supports (Transwell Corning, Inc., Corning, NY). Monolayers were exposed apically to Alexa 488–labeled albumin (Alexa 488-BSA) in the absence and presence of endocytic inhibitors (filipin; dansylcadaverine [DCV]). Transcytotic passage of albumin was monitored for 4 hours by quantitating fluorescence in the basolateral compartment. The mechanism of albumin passage was studied by labeling cell monolayers and cryosections of whole rat lenses for clathrin or caveolin.

Results. The monolayer of cells formed a barrier to the passage of albumin, as shown by the 44% reduction in albumin passage in comparison to nonseeded membranes. Treatment with filipin or DCV reduced the passage of Alexa 488-BSA through lens epithelial cells by 73% and 66%, respectively. Confocal microscopy showed that albumin passage was predominantly transcellular and demonstrated colocalization of albumin with caveolin-1 and clathrin in lens epithelial and fiber cells.

Conclusions. The Transwell apparatus is an excellent system to monitor transport systems across cell monolayers. In this study, rabbit lens epithelial cells formed a confluent monolayer that acted as a barrier to the passive diffusion of albumin. The kinetics of albumin movement across the monolayer and the inhibitor pharmacology suggests that lens cells actively transport albumin from the apical to the basolateral compartment. The inhibitory profile suggests the involvement of caveolae and clathrin-coated vesicles in the transcytotic process. (*Invest Ophthalmol Vis Sci.* 2007;48:1237–1244) DOI:10.1167/iovs.06-0620

There are between 35 and 40 million blind people in the world, with cataract-related blindness accounting for 20 million of these cases.[1] Its incidence is expected to increase by 1.3% by 2050 in the United States as a result of continuous ozone depletion, which could cost $3 billion in cataract operations in the United States, based on 2003 cost levels.[2] To this date, surgical removal of cataractous lenses is the sole known treatment for cataracts. Because of the high cost of cataract surgeries, especially in the United States, where surgery costs range between $2400 and $4930, along with common postsurgical complications, vision researchers are urged to develop alternative methods to prevent or treat the disease.[3] Among innovative treatments, the development of anticataract drugs is a promising one.

Albumin is the major protein of the aqueous and vitreous humors, which are the two ocular media bathing the lens. The macromolecule represents 50% and 40% of the total protein of each medium, respectively. It can bind and transport endogenous ligands, xenobiotics, and therapeutic drugs through the formation of noncovalent complexes at specific binding sites.[4] X-ray analysis of human albumin has revealed the existence of two principal binding cavities in domains IIA and IIIA of the polypeptide.[5] Most drugs can bind in these two preformed and stable high-affinity binding sites. In the past few years, our laboratory has developed an interest in assessing the potential of albumin as a vehicle for the delivery of therapeutically important molecules across the lens epithelium. Delivery systems for albumin, such as albumin-drug complexes,[6] albumin nanoparticles,[7] and albumin-coated liposomes,[8] could be placed in the eye via subconjunctival, intracameral, or intravenous injection or by placing a slow-release device under the sclera.

Albumin is internalized in the lens in vivo, and the capsule is not a barrier to the transepithelial movement of the protein.[9] Besides its relevance as a drug carrier, albumin could be essential for normal lens physiology. Indeed, it was demonstrated recently that a fluorescently labeled fatty acid, when bound to albumin, can be internalized in the lens, where the fatty acid is used for biosynthesis of phospholipids.[10] Recent studies have shown, directly or indirectly, a correlation between abnormal albumin levels and certain types of cataracts. Delcourt et al.[11] reported that patients with low albumin levels have increased risk of cataract. Ha et al.[12] showed that serum albumin can mediate cholesterol efflux from cultured endothelial cells. A similar process could happen in lens epithelial cells, in which case, excessive levels of albumin could trigger a depletion of cholesterol from the lens, resulting in cataractogenesis.[13]

Based on the likely importance of albumin passage into the lens, this study was undertaken to characterize the passage of albumin through lens epithelial cells and to optimize the passage of albumin-drug complexes. To accomplish this goal, a porous filter culture system (Transwell; Corning, Inc., Corning, NY), in which monolayers of lens epithelial cells were grown to confluence, and whole rat lenses were used.

Materials and Methods

Animals

Whole lenses were extracted from 9-week-old rats euthanatized by CO_2 asphyxiation. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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N/N 1003A Rabbit Lens Epithelial Cells

For in vitro experimentation, N/N 1003A rabbit epithelial cells from the cell line established in the laboratory of one of the authors (JR) were used. Studies of the fine structure of the cells revealed that they were comparable to the ultrastructure of epithelial cells from the intact lens.[14] Material relevant to the biochemical properties of the cells confirmed their characterization as lens epithelial cells (McCully RJ et al. *IOVS* 2006;47:ARVO E-Abstract 1987).[15,16]

Measurement of N/N 1003A Rabbit Lens Epithelial Cell Monolayer Resistance

N/N cells were seeded on permeable supports (Snapwell; Corning Inc.) containing 12-mm diameter membranes, each supported by a detachable ring. The cells were grown to confluence and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) (DMEM+FBS) in humidified atmosphere containing 5% CO_2 at 37°C. The medium was changed every 2 to 3 days. Initially, the transepithelial electrical resistance (TER) of cell monolayers was measured beginning at day 5 after seeding until day 23, to determine the optimal time for experimentation. The TER was measured by inserting the detachable ring in a modified Ussing chamber apparatus (model DCV9; Navicyte, San Diego, CA), which was filled on both sides with equal amounts of DMEM+FBS (5 mL) and bubbled with 95% O_2 and 5% CO_2, to ensure constant mixing and maintenance of the pH in each chamber. Electrodes inserted in each chamber permitted the recording of the transepithelial electrical potential and short-circuit current across the monolayer from which the tissue resistance was calculated. The cell monolayer's electrical resistance, a sensitive indicator of the cell monolayer barrier's integrity, was determined at 100-second intervals throughout each experiment.

Characterization of N/N 1003A Rabbit Lens Epithelial Cell Monolayer Tight Junctions

N/N 1003A rabbit lens epithelial cells were grown to confluence for 4 days on a 0.4-μm pore size polyester membrane in 6.5 mm diameter wells (Transwell; Corning, Inc.), in DMEM+FBS in humidified atmosphere containing 5% CO_2 at 37°C. The medium was refreshed on day 2. On the fourth day, the membranes on which the cells had grown were cut from the Transwell insert, washed in phosphate-buffered saline (PBS: 150 mM NaCl, 25 mM NaH_2PO_4 [pH 7.4]) and fixed at room temperature for 30 minutes in 2% (vol/vol) paraformaldehyde in PBS (Electron Microscopy Sciences, Hatfield, PA). After three washes in PBS, the cells were permeabilized in 0.1% (vol/vol) Triton X-100 in PBS for 30 minutes at room temperature. After three washes of 5 minutes each in PBS, the cells were incubated for 1 hour in a solution containing 3% (vol/vol) normal goat serum (Biomeda Corp., Foster City, CA) and 3% (wt/vol) BSA (United States Biochemical Corp., Cleveland, OH) at room temperature. The cells were then labeled with a mouse antibody raised against the tight junction marker ZO-1 (1:200 in PBS; Zymed, South San Francisco, CA) for 2 hours at room temperature. Cells were washed three times in PBS and incubated with an Alexa 594–labeled goat anti-mouse antibody (1:200; Invitrogen-Molecular Probes, Eugene, OR) for 1 hour at room temperature. Finally, the cells were washed in PBS before labeling with a nuclear stain (TO-PRO 3, 1:1000; Invitrogen-Molecular Probes). The membranes were mounted on glass slides (Fluorosave; Calbiochem, San Diego, CA) and observed with an inverted confocal microscope (LSM 510; Carl Zeiss Meditec GmbH, Göttingen, Germany).

Passage of Alexa 488-BSA through a Monolayer of N/N 1003A Rabbit Lens Epithelial Cells

N/N cells were seeded on the permeable filters and maintained as just described. On the fourth day, the cell monolayer was used to assess the passage of fluorescent albumin labeled with the fluorophore Alexa 488 (Invitrogen-Molecular Probes). On the day of the experiments, the cells were washed three times in serum-free DMEM, followed by a 2-hour incubation in DMEM, and a 30-minute incubation in DMEM supplemented with nonlabeled albumin (5 mg/mL) with or without filipin (1.25 μg/mL; Sigma-Aldrich) or dansylcadaverine (DCV; 6.5 μg/mL, Sigma-Aldrich). After this step, the cells were washed three times in DMEM and incubated in one of the following treatment solutions: DMEM, DMEM with filipin, or DMEM with DCV. The volumes in the top (1100 μL) and bottom (200 μL) compartments were chosen so that their heights would match, to prevent the creation of a hydrostatic force across the cell monolayer. Four microliters of Alexa 488-BSA (2.5 mg/mL) were added to the top compartment, to initiate the experiment, and the passage of the fluorescently labeled protein into the lower compartment was assessed at time 0 and after 1, 2, 3, and 4 hours. A filter insert without cells was monitored in parallel to verify free passage of albumin across the porous filter membrane. The experiment was repeated four times, and the results shown represent an average of four repetitions.

Confocal Microscopy of Intracellular Passage of Albumin through N/N 1003A Rabbit Lens Epithelial Cells

Confocal microscopy was used to view the passage of fluorescently labeled albumin across monolayers of N/N rabbit cells grown on Transwell supports. Cell monolayers that had been used to assess the passage of Alexa 488-BSA were washed three times with DMEM followed by a 4-minute incubation on ice in DMEM adjusted to pH 2.0 with HCl to remove surface bound Alexa 488-BSA. The cells were washed three times in PBS and fixed in 2% (vol/vol) paraformaldehyde in PBS for 30 minutes. The cell membranes were permeabilized with a 5-minute treatment in 0.1% (vol/vol) Triton X-100 in PBS. The nuclei were stained (TO-PRO 3; 1:1000 in PBS; Invitrogen-Molecular Probes) for 30 minutes at room temperature in the dark, followed by three washes in PBS. Transwell supports on which the cells had grown were cut and mounted (Fluorosave; Calbiochem) on a glass slide, before visualization with an inverted fluorescence microscope (LSM 510; Carl Zeiss Meditec GmbH).

Albumin–Caveolin-1 and Albumin–Clathrin Colocalization study in N/N 1003A Rabbit Lens Epithelial Cells and in the Whole Rat Lens

To study the mechanism of passage of albumin, we looked for the potential colocalization of albumin with caveolin-1 or with clathrin in lens cells by using two systems: the N/N lens epithelial cell monolayer model and the whole rat lens model. The decision to use two different species (rabbit and rat) and two different culture systems (cells in culture and whole lens, respectively) was made to strengthen results found in both systems and species. For lens cells in the culture model, the cells were prepared as described in the section on Passage of Alexa 488-BSA through a Monolayer of N/N 1003A Rabbit Lens Epithelial Cells. After 2 hours in the presence of Alexa 488-BSA, the cells were washed with DMEM and treated with DMEM at pH 2.0 before fixation in 2% (vol/vol) paraformaldehyde for 30 minutes. After treatment in 0.1% (vol/vol) Triton X-100 for 30 minutes and extensive washing with PBS, the cell monolayers were incubated for 1 hour in blocking buffer (3% [wt/vol] BSA, 3% [vol/vol] normal goat serum), followed by incubation in blocking buffer containing monoclonal mouse IgG against human caveolin-1 (5 μg/mL; BD Biosciences, San Jose, CA) or monoclonal mouse IgG against human clathrin (6 μg/mL; Affinity Bioreagents, Golden, CO), for 2 hours, and washed with PBS. Primary antibodies were visualized after a 1-hour incubation with blocking buffer containing Alexa 633 goat anti-mouse IgG (10 μg/mL; Invitrogen-Molecular Probes), followed by washing with PBS. Identical treatment with normal mouse serum showed no fluorescence for Alexa 633 (results not shown).

For the whole rat lens model, lenses were removed from euthanatized 9-week-old male rats, fixed immediately in 0.8% (vol/vol) paraformaldehyde solution in PBS for 24 hours, followed by incubation in

10% (wt/vol), then 20% (wt/vol), then 30% (wt/vol) sucrose solution in PBS over a 24-hour period. The lenses were fast frozen in isopentane, and sectioned on a cryostat (CM 3050 S; Leica Microsystems, Nussloch, Germany) into 16-μm sections. After treatment with 1% (vol/vol) Triton X-100 for 30 minutes, lens sections were treated according to the same protocol as used for lens epithelial cells in culture, except that lens sections were also incubated in blocking buffer containing polyclonal rabbit IgG against rat albumin (11.4 μg/mL, Research Diagnostics, Inc., Concord, MA) for 2 hours, followed by a 1-hour incubation in blocking buffer containing Alexa 546 goat anti-rabbit IgG (10 μg/mL; Invitrogen-Molecular Probes). Visualization was performed with an inverted confocal microscope equipped with an HeNe laser (543 and 633 nm; LSM 510; Carl Zeiss Meditec GmbH).

Western Blot Analysis of Whole Rat Lens

Whole lenses were homogenized in 1 mL $MgCl_2$-Tris buffer (20 mM $MgCl_2$, 50 mM Tris [pH 7.5]) and 1 μL protease inhibitor (1:1000 in lysis buffer consisting of 20 mM Tris, 0.5 mM EDTA, 0.5 mM EGTA, and 0.5% [vol/vol] Triton X-100). The solution was then sonicated before the protein content was determined with a Bradford assay (Bio-Rad, Richmond, CA). Twenty microliters of lens homogenate solution was withdrawn to prepare tubes containing 50 μg of protein plus 5 μL of 5× Coomassie dye. Tubes were boiled for 5 minutes. Proteins from each tube were separated by SDS-PAGE (120 V, 70 minutes; Mini-Protean-3 Cell; Bio-Rad, Hercules, CA) using 12% resolving gels. The resolved proteins were transferred to nitrocellulose membranes with a minitrans blot apparatus (350 mA, 2 hours; Bio-Rad). Membranes were blocked for 1 hour in 5% (wt/vol) nonfat dry milk on a shaker at room temperature. They were then probed with a polyclonal rabbit IgG against rat albumin (5 μg/mL, RDI, Flanders, NJ) in 5% (wt/vol) nonfat dry milk overnight at 4°C on a shaker. Membranes were washed three times for 10 minutes in Tris-EDTA-NaCl (TDN: 50 mM NaCl, 10 mM Tris, 2 mM EDTA [pH 7.4]) and incubated with a goat anti-rabbit antibody conjugated horse radish peroxidase (1:5000, Promega, Madison, WI) in 5% (wt/vol) nonfat dry milk for 2 hours at room temperature on a shaker. Membranes were washed again three times for 10 minutes in TDN before band development by chemoluminescence reaction (ECL; Pierce, Rockford, IL). Controls were obtained by blotting the membranes with normal rabbit serum (5 μg/mL; Biomeda) instead of the primary antibody.

All commonly used laboratory reagents were of reagent grade or higher.

Statistical Analysis

Passage of Alexa 488-BSA across N/N cell monolayers was kinetically analyzed (SigmaPlot 2000, ver. 6.0; Systat Software, Inc., Richmond, CA). Data sets were fitted by a standard three-parameter function for the exponential rise to a maximum or to a user-defined function that included the initial function plus a linear regression. The initial data set for the blank filters was fitted with no constraints. Based on these observations, the other data sets were fitted with constraints that maintained logical outcomes (e.g., maximum diffusion rate could not exceed that derived for the blank filter). Data represented are the mean ± SEM of results in three or four independent repetitions.

Results

Measurement of N/N 1003A Rabbit Lens Epithelial Cell Resistance

The N/N cell monolayer resistance varied little over the course of the 4-week measurement. It became stable at day 5 at a value of 22 Ù/cm^2 with observations not appreciably different through day 23. This electrical resistance value is similar to values obtained with other epithelial cell monolayers. CaSki endocervical epithelial cells and human ectocervical epithelial cells were reported to have transepithelial electrical resistances of 10 ± 2 and 18 Ù/cm^2, respectively, after 5-days' growth on filters in culture medium.[17] Even though these resistance levels were lower than some other cell types, the cervical epithelial cells were able to restrict the free movement of solutes through their intercellular space.[17]

Characterization of N/N 1003A Rabbit Lens Epithelial Cell Monolayer Tight Junctions

The presence of the marker of the tight junction protein ZO-1 along the edge of cells in a confluent monolayer suggested that the N/N 1003A rabbit lens epithelial cells were joined by tight junctions (Fig. 1). ZO-1 was in a punctate distribution, similar to the distribution in mouse lens epithelial cells,[18] but not as abundant as in epithelial cell monolayers from other tissue types with greater electrical resistance.[19] Even though the presence of tight junctions does not completely eliminate the possibility of diffusion between cells as shown by Rae et al.,[20] these data strongly suggested that the N/N cell monolayers were tight and confluent and could form a barrier to the passive diffusion of albumin.

Passage of Alexa 488-BSA through a Monolayer of N/N 1003A Rabbit Lens Epithelial Cells

Results presented in Figure 2 demonstrate that N/N cells provide a biological barrier to the free diffusion of dissolved macromolecules and suggest that these cells facilitate passage of albumin from the apical to the basolateral compartment. The apical and basolateral surfaces of the cell monolayer itself are believed to correlate with the apical and basolateral compartments of the Transwell. Previous observations by Reddan et al. showed that rabbit 1003A N/N cells cultured on tissue culture dishes produce a basement membrane on the bottom of the culture dish, which defines the basal surface of the cells. The apical surface of the cells faces the upper culture medium (unpublished data).

When albumin was placed in the apical compartment of the permeable filter apparatus (Transwell; Corning, Inc.), the rate of appearance of fluorescence in the basolateral compartment followed a saturation function, as expected for a diffusion-limited process, with a half-life of ~2.4 hours. The N/N cell monolayer (Fig. 2, Cells) formed a barrier to diffusion that was fitted by a more complex function that included the addition of a linear component. The solid line associated with these data points represents a saturating function with the same half-life as observed for the blank filter and a maximum of only 167 arbitrary units. Of importance, the additional linear component with a slope of 262 arbitrary units per hour was necessary to fit the data set. This outcome suggests an active transepithelial transport process. To test this hypothesis, monolayers were pretreated with either DCV to inhibit the formation of clathrin-coated vesicles or with filipin to inhibit caveolae formation. Both treatments resulted in a significant reduction in the appearance of fluorescence in the basolateral compartment. A diffusion-dependent component could not be clearly resolved by this analysis, and the linear component was reduced to rates of 70 and 86 arbitrary units per hour, respectively. Together, these results strongly suggest that albumin was transcytosed through N/N lens epithelial cells at least partially by an endocytic mechanism involving both clathrin-coated vesicles and caveolae.

Confocal Microscopy Images Showing Intracellular Passage of Albumin through N/N Lens Epithelial Cells

The kinetics of albumin transport along with inhibition of passage by filipin and DCV strongly suggests that albumin

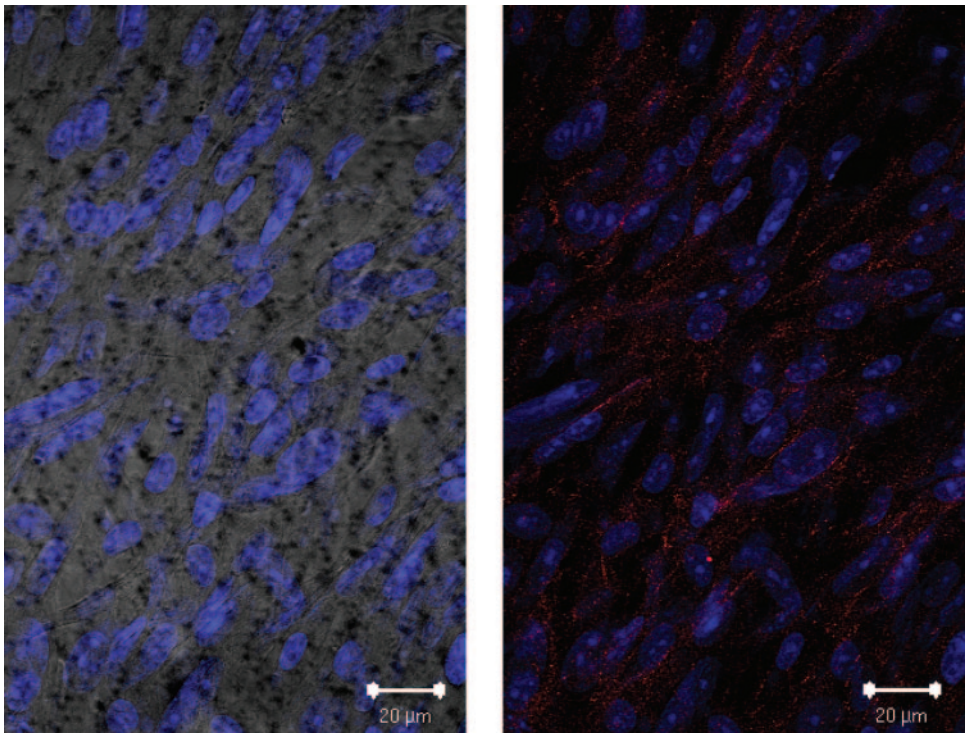

FIGURE 1. N/N cells labeled with ZO-1 tight junction marker and a nuclear dye. N/N lens epithelial cells were grown on a permeable membrane for 4 days. The monolayer was then labeled to determine the presence of tight junctions with the molecular marker ZO-1 (*red*). The cells were also labeled with the nuclear dye (*blue*) to help distinguish individual cells. *Left*: differential interference contrast image; *right*: fluorescence image of the same sample. A negative control showed no fluorescence (results not shown).

passes through the cell monolayer via a transcytotic mechanism. To test this hypothesis further, we observed N/N cell monolayers on the membranes filters by confocal microscopy. These images showed that the Alexa 488-BSA was in all levels of the cells (Fig. 3). This was determined by comparing the position of Alexa 488-BSA (green) with the nuclear dye (blue; TO-PRO 3; Invitrogen-Molecular Probes), looking at different planes of *z*-stack images of the cell monolayer. Albumin was present in the basolateral region of the cells (Fig. 3A). Going up through the cell (Figs. 3B, 3C), the fluorescence decreased, even though the confocal image is still within the cell as shown by nuclei labeling. A sagittal cut through the cell layer shows the coplanar presence of albumin and a cell nucleus (Fig. 3D). Based on examination of additional images, (data not shown) albumin labeling was not restricted to the edge of the cells (i.e., the paracellular space), which would suggest that its passage is not exclusively extracellular. These results strengthen the hypothesis that albumin passage is transcellular rather than paracellular. The confocal images also confirm that the N/N cells grew as a monolayer on the culture support.

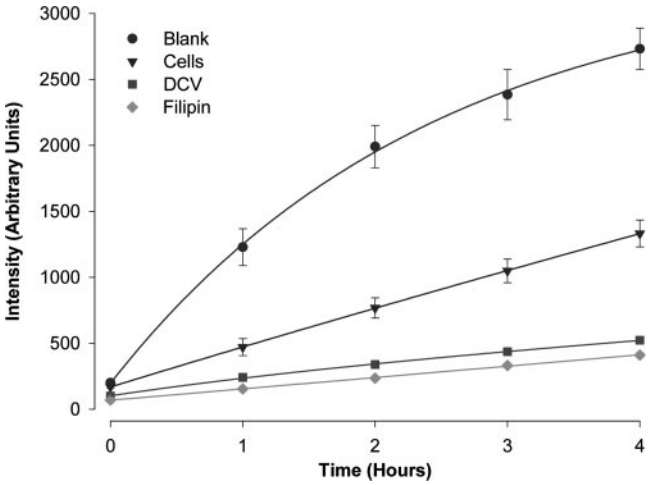

FIGURE 2. Transcytosis of fluorescently labeled albumin through a monolayer of N/N cells grown on a permeable support (Transwell; Invitrogen-Molecular Probes). The passage of albumin was assessed through nonseeded membranes (*circle*), seeded with nontreated N/N cells (*triangle*), seeded with N/N cells treated with DCV (which is an inhibitor of clathrin-mediated endocytosis, *square*), or seeded with N/N cells treated with filipin (which is an inhibitor of caveolin-mediated endocytosis, *diamond*). Samples from the lower compartment of the apparatus were analyzed for fluorescence every hour for 4 hours. The graph represents the data for the passage of albumin added over time. Bars, SEM.

Albumin and Clathrin Colocalization in N/N Rabbit Lens Epithelial Cells in Culture

Passage of albumin through an N/N cell monolayer suggested the involvement of clathrin and caveolin in the process, since treatments with DCV or filipin significantly reduced the appearance of fluorescence in the basolateral chamber. To test further for a role of clathrin and caveolin in the passage of albumin through N/N cells, an immunofluorescence technique was used to identify sites of colocalization between albumin and clathrin, and albumin, and caveolin-1. Figure 4 shows a fluorescent image in which cells had been incubated with both Alexa 488-BSA (green fluorescence) and an antibody against clathrin (blue fluorescence). A profiling path (arrow) was drawn in Figure 4A to characterize the areas where albumin (Fig. 4B) and clathrin (Fig. 4C) were labeled. In Figure 4B, the albumin profile shows peaks of greater fluorescence intensity, which correspond to locations where albumin was present. Similarly, on Figure 4C, peaks of greater fluorescence on the profile show locations of clathrin. Structures showing colocalization correspond to areas containing peaks for both albumin and clathrin and are indicated with arrows. Because it was not always possible to distinguish different colors within Figure 4A, the profiling pathway option of the microscope system software (LSM 5 Image Software; Carl Zeiss Meditec GmbH) was used to determine colocalization. These data show for the

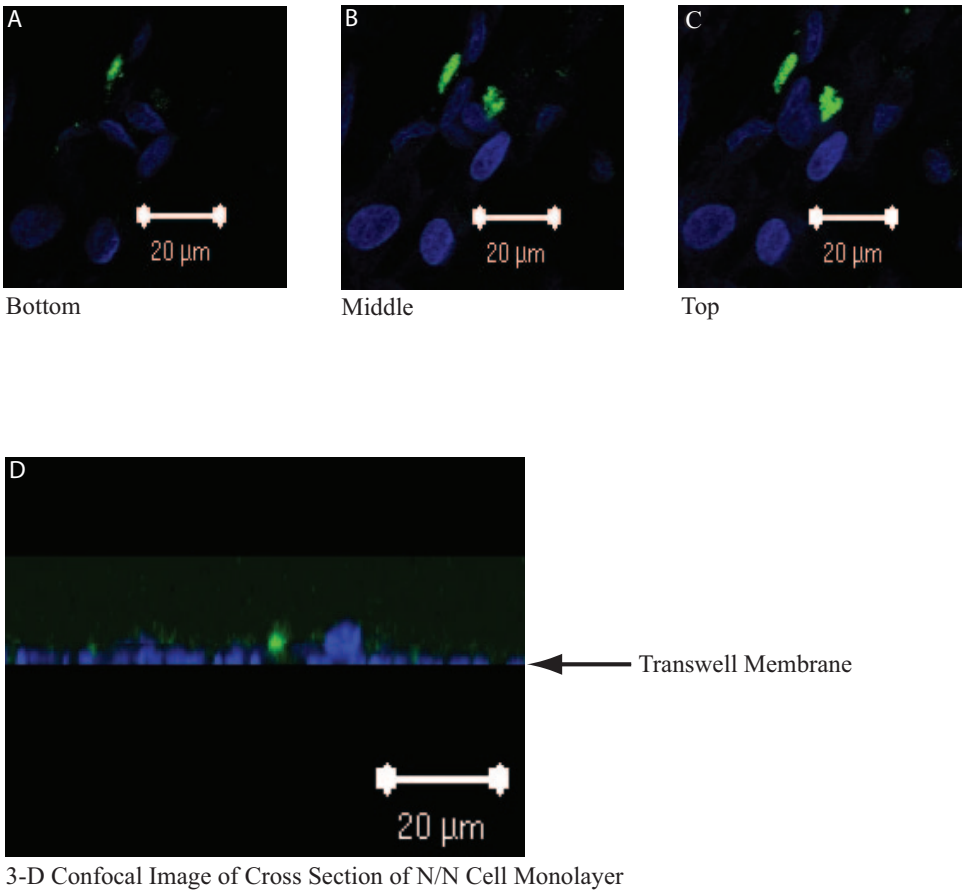

FIGURE 3. Sagittal and transversal sections of N/N cell monolayer showing the intracellular passage of Alexa-488 albumin. N/N cells were grown as a monolayer for 4 days on a permeable insert (Transwell; Invitrogen-Molecular Probes). On the experimental day, fluorescently labeled albumin (*green*) was added to the top chamber of the apparatus, to study its passage through the cell monolayer. The cells were stained with a nuclear dye (*blue*), to visualize the localization of nuclei in the cell monolayer. (**A**), (**B**), and (**C**) represent different planes of the same cell monolayer relative to the nucleus, going from the basolateral side of the cell, which was close to the membrane, toward the apical side of the cell. (**D**) Sagittal cut of the permeable membrane, showing the presence of *blue* nuclei and labeled albumin in the same plane of the cell monolayer.

first time in lens epithelial cells the colocalization of albumin with clathrin. Similar data were collected and showed colocalization between albumin and caveolin in N/N cells (data not shown). Together, these results reinforce the hypothesis that albumin is internalized into lens cells via clathrin- and caveolin-mediated endocytosis.

Western Blot Analysis of Native Albumin in Whole Rat Lens

To ascertain that albumin was indeed found in the intact lens, lens homogenates were analyzed by Western blot analysis. A band at approximately 65 kDa developed on the film, which corresponds to the expected mobility of albumin (Fig. 5). When probed with normal rabbit serum, no bands were observed (results not shown). These results demonstrate that albumin is present in rat lens. Subsequently, the colocalization of albumin with clathrin and caveolin in whole rat lenses was assessed.

Albumin and Caveolin Colocalization in the Whole Rat Lens

Previous studies have shown the presence of clathrin and caveolin in lens tissue,[21–23] and Western blot analysis has shown the presence of native albumin in the lens tissue. In Figure 6, profiles for both albumin and caveolin-1 contained peaks of both fluorophores. These profiles showed that in certain parts of the lens, illustrated by vertical arrows, there was colocalization of peaks. Because it was not always possible to distinguish different colors within Figure 6A, the profiling pathway option of the microscope system software (LSM 5 Image; Carl Zeiss Meditec GmbH) was used to determine colocalization. The colocalization occurred not only in epithelial cells but also in fiber cells that can be visualized by confocal microscopy deep in the epithelial cell layer. This colocalization of peaks supports the earlier observations that albumin was traveling via an endocytic mechanism, which included caveolae. Similar colocalization results were obtained with albumin and clathrin (data not shown), which supports a role for clathrin-mediated endocytosis as another means of vesicular transport of albumin through lens cells. Thus, whether in lens cells in culture or whole tissue and in the passage of exogenous or endogenous protein, albumin colocalized with two major proteins that are involved in endocytosis: clathrin and caveolin-1.

DISCUSSION

Presently, the only treatment for cataracts is surgery. Unfortunately, the procedure is expensive in developed countries, where it has become a common practice, and it is difficult to access in developing countries. Consequently, researchers in ophthalmology are urged to develop other techniques for the treatment of cataracts. Recently, Lin et al.[24] have shown the overexpression of superoxide dismutase 1 (SOD1) in the intact lens, which resulted in attenuating the effect of H_2O_2 oxidative damage. This observation opens new avenues in the development of potential methods for the treatment of cataracts. Indeed, one can envision a delivery system for DNA coding for important antioxidant enzymes such as SOD1. Albumin is believed to be one of the best candidates for such a delivery system, as it was shown to be an effective tool in the delivery of drugs in vivo. The strong interaction of cationic forms of albumin to negatively charged cell surfaces[25] and to its cell receptors gp60[25] favor the internalization of albumin proteins

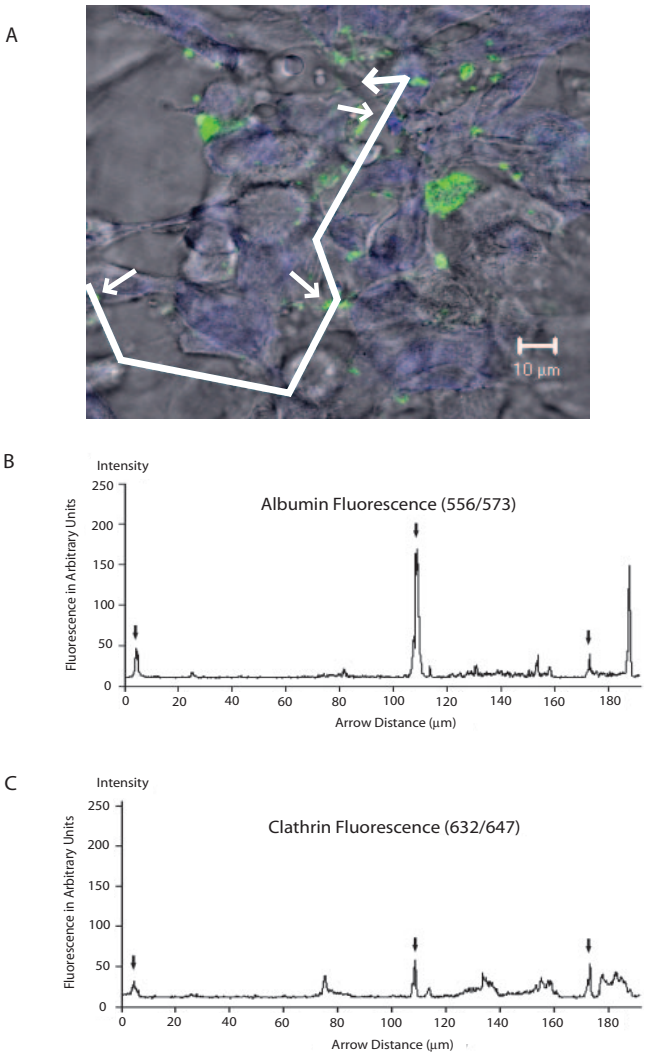

FIGURE 4. Colocalization of clathrin and albumin in N/N rabbit lens epithelial cells. N/N cells were grown on a permeable support (Transwell; Invitrogen-Molecular Probes), treated with fluorescently labeled albumin and with an antibody against clathrin. The profiling path (*arrow*) on the confocal image was translated into the corresponding profiling paths for albumin (**B**) and clathrin (**C**) labeling (*left* to *right*). (**B**) Albumin profile showing peaks of greater fluorescence intensity and correspond to locations where albumin was present. (**C**) Peaks of greater fluorescence on the profile showing locations where clathrin was present. Structures showing colocalization are designated with *vertical arrows* in (**B**) and (**C**) and by *white arrows* in (**A**). *Short arrows* in (**A**, *left* to *right*) correspond with *arrows* in (**B**) and (**C**) (*left* to *right*).

into cells. Thus, to optimize passage and recycling of therapeutic molecules such as DNA across the lens, it is critical to understand the mechanism of passage of albumin through lens cells.

It is also important to understand better the role(s) of albumin in lens metabolism, as different studies have directly or indirectly suggested a role of albumin in cataract formation. Delcourt et al.[11] have shown that low levels of albumin are associated with a 50% increase in the risk of developing cataracts in people between ages 60 and 95. In another study,[12] it has been shown that human serum albumin mediates cholesterol efflux from cultured endothelial cells. If this observation were also true in lens epithelial cells, along with cholesterol insufficiency, which underlies lens cell proliferation leading to eventual cataract formation in Shumiya cataract rats,[13] then high levels of serum albumin could also increase the risk of cataract formation.

In previous studies, albumin passage through different epithelial cells has been reported to take place through a variety of paths. For instance, Kim et al.[26] suggested that transcytosis of albumin from apical to basolateral surfaces of alveolar epithelium involves both caveolae and clathrin-coated vesicles. Monks and Neville[27] showed in lactating mammary gland that albumin passage was affected by inhibitors specific to clathrin-coated vesicles, but not by filipin. In addition, the study showed no colocalization between albumin and either caveolin-1 or -2.

Filipin was chosen because it is known to bind sterols such as cholesterol, which is an essential component of caveolae and which appears to be essential to maintain the structural integrity of this vesicular complex.[28] Furthermore, Schnitzer et al.[29] showed that filipin prevents receptor clustering within caveolae and endocytosis through the noncoated vesicular pathway in bovine lung endothelial cells, without disrupting endocytic events leading to degradation, which are mediated by the clathrin-coated pathway. DCV was chosen because it has been shown to prevent clathrin-mediated endocytosis.[30]

The objective of the present study was to characterize the mode of internalization of albumin in lens epithelial cells by using a permeable filter apparatus (Transwell; Corning, Inc.). In the present study, we looked specifically at the passage from apical to basolateral surfaces of the epithelial cells, which in the lens would correspond to movement from the fiber cells to the capsule via the epithelial layer. The results could be used to assess drug delivery system involving the use of albumin, and further work should be considered to establish the characteristics of the reverse transport mechanism for the delivery of drug from the aqueous or vitreous humors into the lens.

After growing a confluent monolayer of N/N rabbit lens epithelial cells that expressed a marker of tight junctions, ZO-1, at cell-to-cell contact points, we assessed the kinetics and pharmacology of transepithelial albumin movement. First, comparison of the protein passage in the absence (blank) or presence of cells demonstrated that the cell monolayer forms a barrier that separates the two compartments. The presence of a lens epithelial cell monolayer reduced by half ($P < 0.05$) the appearance of albumin in the basolateral chamber in the 4-hour test period. The kinetic analysis suggested an ongoing active transport process, and treatment of the monolayers with either

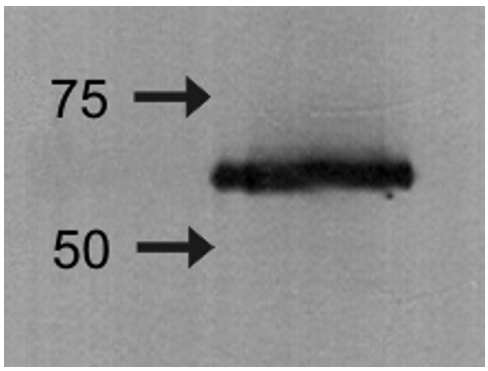

FIGURE 5. Western blot showing immunoreactivity to native albumin in whole rat lens. Proteins of whole rat lens homogenates were analyzed by Western blot analysis to show the presence of albumin. A band at approximately 65 kDa developed on the film, which corresponds to the molecular weight of albumin. *Arrows*: molecular size markers.

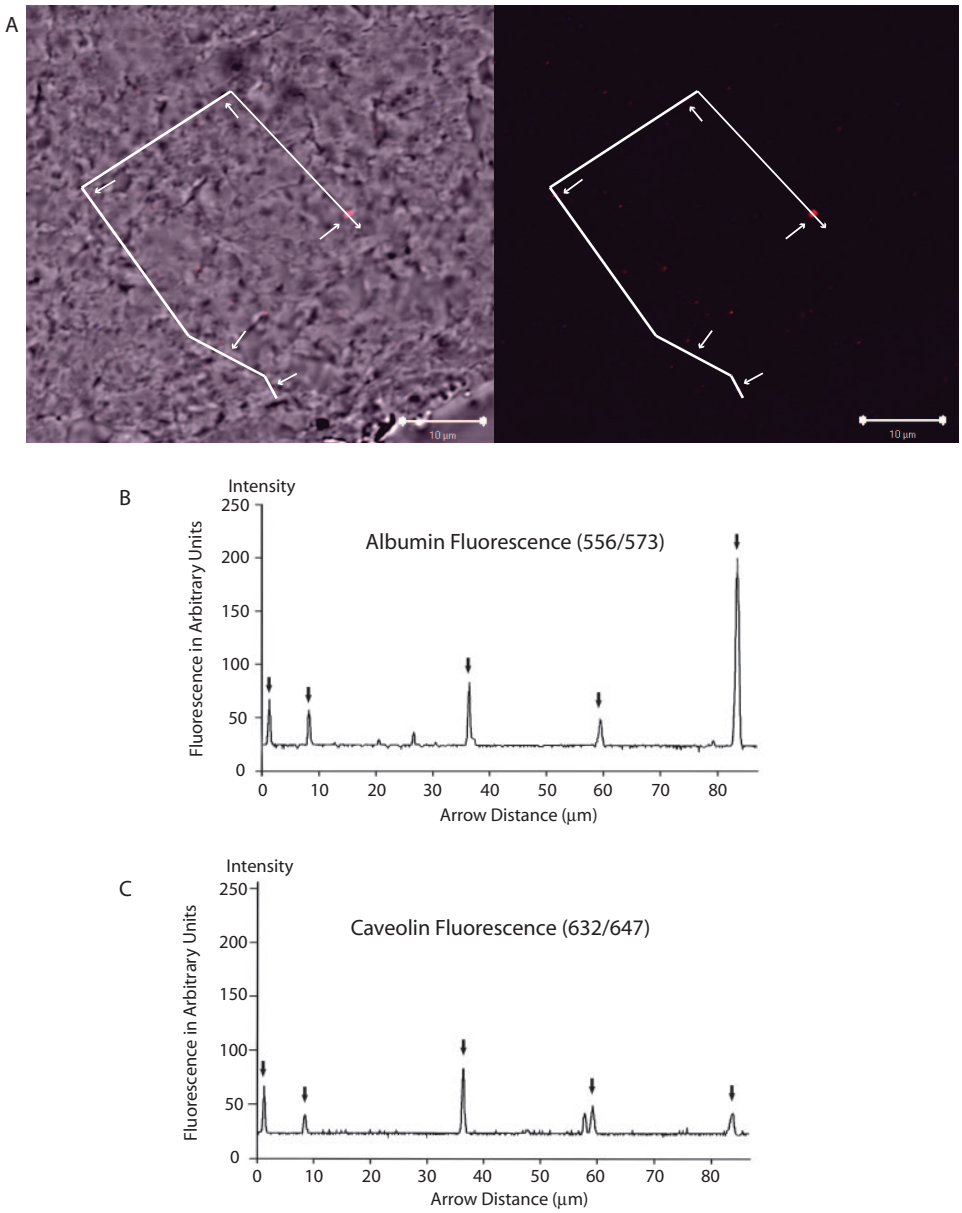

FIGURE 6. Colocalization of caveolin-1 and albumin in whole rat lenses. Whole rat lenses were fast frozen in isopentane and cryosectioned before labeling with antibodies against albumin and caveolin. The profiling path (*white arrow*) on the (**A**) confocal image was translated into the corresponding profiling paths for albumin (**B**) and caveolin (**C**, *left* to *right*). (**B**) Albumin profile showing peaks of greater fluorescence intensity, which correspond to locations where albumin was present. (**C**) Peaks of greater fluorescence on the profile show where clathrin was present. Structures showing colocalization are designated with *vertical arrows*. (**A**, *short arrows*) correspond with *arrows* in (**B**) and (**C**) (*left* to *right*).

DCV or filipin revealed the true barrier function of the lens epithelium, since appearance of albumin in the basal compartment was reduced to less than 15% of the diffusion-limited amount.

The results obtained when cells were treated with either filipin or DCV showed a significant decrease in the amount of albumin passage through the cell monolayer. That the rate of albumin movement was reduced by each treatment by more than two thirds suggests that a common pathway inhibited by both compounds accounts for at least a portion of the transepithelial movement. These results are consistent with the involvement of clathrin-coated vesicles and caveolae in transcytosis of albumin across lens epithelial cells.

Results of confocal microscopic studies provide strong evidence to support the hypothesis that clathrin and caveolin participate in albumin transcytosis. First, confocal microscopy (Fig. 3) showed the presence of fluorescently labeled albumin in discrete "packages" around the cell nucleus. The fluorescence was not consistent with a paracellular distribution. Furthermore, dual labeling with antibodies raised against either albumin and clathrin or albumin and caveolin-1 revealed their colocalization.

To reduce possible artifacts due to the use of labeled bovine albumin and the use of an established cell line, the presence of endogenous albumin in whole rat lenses was determined. Once it was established by Western blot analysis that nontreated rat lenses contain albumin, its colocalization with clathrin and caveolin in intact whole rat lenses was studied. The results were consistent with the earlier observations obtained with N/N cells.

This study shows for the first time that albumin passage through the lens cells occurs via endocytic mechanisms that are clathrin- and caveolin-mediated. The results should provide the basis for further characterization of cellular mechanisms that might be involved in the etiology and treatment of cataracts. In addition, future studies of these transport systems should increase our understanding of normal macromolecular passage through the lens and will hopefully assist in the development of optimal methods for the delivery of therapeutic molecules to the lens.

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