Tight Junctions in the Lens Epithelia of Human and Frog: Freeze-fracture and Protein Tracer Studies

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For the first time, the existence of zonulae occludentes in the lens epithelia of human and frog has been demonstrated, using a “double mounting” method in freeze-fracture transmission electron microscope (TEM). The physiologic barrier function of zonulae occludentes in frog lens epithelium is determined by a “wash out” procedure in the protein tracer studies. It was found that within various time intervals of washing, horseradish peroxidase (HRP) was consistently restricted to the location of membrane fusions (zonulae occludentes), as seen with thin-section TEM. This corresponds to the location of zonulae occludentes found in the freeze-fracture studies. Thus, these data strongly suggest that there are zonulae occludentes in the frog lens epithelium and that these structures do provide a barrier function for the transepithelial diffusion of HRP with a molecular weight of 40,000 daltons.


It has been shown that the anterior surface of the lens has a greater barrier to the diffusion of substances into the lens cortex than does the posterior surface.1 This, the monolayer of lens epithelium, which exists only at the anterior surface of the lens, has been speculated to be the site of this physiologic barrier. Zonulae occludentes (tight junctions) found in various epithelia are believed to provide a diffusion barrier to substances in the extracellular pathway.2 However, the existence of zonulae occludentes between the lens epithelial cells in various species is uncertain.3 Most recently, Goodenough et al,4 using a freeze-fracture technique, identified the zonulae occludentes between the lens epithelial cells of chick, but not in the mouse. Using the same technique, Benedetti et al5 were also unable to find zonulae occludentes in the bovine lens epithelium. This raises a question as to whether the zonula occludens is a structure commonly present in the lens epithelium of most vertebrates. The present study demonstrates for the first time the presence of zonulae occludentes between lens epithelial cells of human and frog, using a “double mounting” method in freeze-fracture electron microscopy. Furthermore, by using a “wash out” procedure in the protein tracer experiments, our preliminary results strongly suggest that the zonulae occludentes in the lens epithelium of frog do serve a barrier function for the transepithelial diffusion of the protein (horseradish peroxidase) with a molecular weight of 40,000 daltons.

Materials and Methods

Adult frog (Rana pipiens) lenses and adult human lenses with slight opacities obtained from routine cataract surgery were used in this study.

Freeze-fracture TEM

These lenses were fixed in 2.5% glutaraldehyde-0.1 M cacodylate buffer at pH 7.3 immediately after lens extractions. The Cooperative Cataract Research Group photographs7 of human lenses were taken within about 5 min after they were placed in the fixative. Each lens was fixed at room temperature for a total of 2 hrs, then transferred into 0.1 M cacodylate buffer at pH 7.3. The anterior half of the lens, which contains the epithelium, was obtained by making a cut around the posterior margin of the equator. It was infiltrated with 25% glycerol in 0.1 M cacodylate buffer, pH 7.3 for 1-2 hrs at room temperature. In some cases, in order to obtain better cryoprotection, the remainder of the lens nucleus and some inner portions of cortex within the anterior half of the lens were removed by forceps at the beginning of the infiltration process. Small rectangular blocks of lens tissues containing capsule, epithelial cells, and small parts of the outer cortex from central and peripheral zones of the epithelium were dissected. In order to obtain good replicas of lens epithelium more easily
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and reproducibly, a new method, designated as “double mounting” technique, has been developed for this study. Two small rectangular blocks of lens tissue are mounted on the tissue holder, so that their capsule surfaces are facing each other and touching. The tissue is oriented so that cross sections of the lens epithelium can be obtained. Thus, two monolayers of lens epithelium are obtained in the middle of a single good replica. Also, the epithelium and capsule are physically stabilized by this arrangement. Freezing of the tissues was done initially in liquified Freon 22, then stored in liquid nitrogen. Freeze-fracture was performed with a Balzers 301 freeze-etch device. Fracturing was carried out at —105 C under a vacuum of at least 1 X 10^-6 torr to avoid condensation of water vapor on the specimen surface. The fractured surfaces were coated with platinum and carbon. Replicas were cleaned in a mixture of 25% buffered glycerol and Clorox bleach followed by Clorox only to remove adherent lens tissues, rinsed in distilled water, mounted on uncoated 200 mesh grids, and examined in a Philips 301 transmission electron microscope.

We have found that the “double mounting” method for making replicas of lens epithelium has several advantages: (1) It greatly increases the chances of making good replicas of lens epithelium by mounting two blocks of lens tissue so that the two monolayers are arranged in the middle of the replica. This greatly avoids the possible loss or damage of the epithelium due to possible breaks of the replica around the edges, which often happens during the cleaning process. (2) This procedure, along with the cross-sectional orientation of the epithelium, makes it relatively easy to identify the lens epithelial cells. This is particularly important in the equatorial regions, where both epithelial cells and fiber cells contain organelles.

Tracer Studies

The tracer studies were conducted in ten frog lenses in a “wash-out” procedure to compare with a conventional “nonwash” procedure.

The “wash-out” procedure is as follows: Injection of the protein tracer horseradish peroxidase (HRP) was done by a modified procedure of Gorthy et al. HRP (type II) (Sigma Chemical Co., St. Louis, MO) solution was prepared in a concentration of 1% (w/v) in 70% TC-199 and was injected into the freshly anucleated eye. Injection (0.05 cc) was made into the anterior chamber through the cornea by using a 30-gauge needle. After 15 min of enzyme incubation in the eye at room temperature, the lens was removed and was washed in 10 ml of 70% TC-199 in a vial, with one quick change within the first minute followed by two additional changes within various time intervals of washing (eg, 2, 7.5, 10, 15, and 20 min). The lens was fixed in 2.5% or 4% glutaraldehyde-0.1 M cacodylate buffer pH 7.3 for 1 hr at room temperature. Anterior lens epithelium with a small portion of cortical fibers was dissected out and fixed for one additional hour. Lens tissue blocks were chopped into 20 micron and 40 micron thick sections with a TC-2 tissue sectioner (Ivan Sorvall, Newton, CT). Thick sections of lens tissue were then collected in 0.1 M cacodylate buffer, pH 7.3 for cytochemistry. Cytochemical demonstration of HRP was done according to Graham and Karnovsky as modified by Malmgren and Olsson. The DAB medium contained 20 mg 3,3'-diaminobenzidine-tetrahydrochloride (Sigma Chemical Co.), 0.1 ml of 1% H2O2 in 9.9 ml of 0.1 M cacodylate buffer, pH 5.1. Tissue sections were incubated in water bath at 37 C for 1 hr. After incubation, sections were rinsed three times with 0.1 M cacodylate buffer, pH 7.3. Twenty micron thick sections were mounted on glass slides, dried on a hot plate, covered with immersion oil and cover slips, and examined with a light microscope to determine the cytochemical reaction. When a cytochemical reaction was obtained, 40 micron thick sections were processed for ultrastructural cytochemistry. These sections were fixed in 1% OsO4-0.1 M cacodylate buffer, pH 7.3 for 1 hr at room temperature. They were rinsed in buffer twice and stained en bloc with 0.5% uranyl acetate in 0.15 M NaCl for 30 min at room temperature, rinsed in buffer, dehydrated through graded ethanol (30, 50, 70, 95, and 100%), 15 min for each change and an additional two changes for 100% ethanol, rinsed in propylene oxide, and embedded in Epon 812 or Spurr’s resin. Thick (1.5 micron) and thin sections were cut with a Porter-Blum MT-2 ultramicrotome. Thick, unstained sections were examined with phase contrast in a Zeiss photomicroscope. Thin sections, either unstained or stained with Reynold’s lead citrate were examined in a Philips 301 electron microscope equipped with a goniometer stage.

Immersion of freshly isolated frog lenses in HRP solution (1-3 mg HRP/ml 70% TC-199) for 15 min was also performed in the “wash out” tracer experiments. Cytochemical procedures were same as above.

The “nonwash” experiments: The injection procedure was the same as that in the “wash-out” procedure, except there was no wash after 15 min of enzyme incubation. The eye was fixed in 4% or 2.5% glutaraldehyde-0.1 M cacodylate buffer for 30 min at room temperature. The lens was removed and fixed for an additional 1 1/2 hrs. The lens was dissected and processed for cytochemistry as that described in the “wash-out procedure”. The “non-wash” lens serves as a control for the “wash-out” lens.
Results

Freeze-fracture

Observations were made on many replicas of lens epithelia of human and frog in which the cells were fractured in a cross-sectional orientation, as shown in Figure 1A and 2A. The monolayer of the epithelial cells can be identified easily. These epithelial cells contain numerous organelles and some areas of fracture faces of membranes. The structures of zonulae

Fig. 1. A, Freeze-fracture replica of human lens epithelial cells in a cross-sectional orientation. Epithelial cells (E) expose numerous small organelles and some areas of fracture faces of cell membranes. Grooves of zonulæ occludentes are found at the apical end of the epithelial cell (shown within the rectangle). C = Capsule, F = Fiber cell. Bar = 1 μm; B, High magnification of the zonulæ occludentes shown in the rectangle of Figure 1A. Continuous anastomosing grooves are evident on the E-face membrane (arrow heads). A gap junction (arrow) with a small aggregate of particles and pits is in close association with the zonulæ occludentes. P = P-face, E = E-face. Bar = 0.1 μm.
occludentes can be determined on the P and E fracture faces of the membranes.

It was found that these zonulae occludentes are always located between the lateral membranes of epithelial cells with close proximity to the apical end (ie, close to fiber cells) of the cells in human (Fig. 1A) and in frog (Fig. 2A). Ultrastructurally, the zonulae occludentes in the human lens epithelium are characterized by a few continuous anastomosing grooves (furrows) on the E-face of the membrane (Fig. 1B). Figure 2B shows the zonulae occludentes in the frog lens epithelium that are characterized by a number of continuous anastomosing strands as seen on the E-face. These two structural configurations of freeze-fractured zonulae occludentes have been well documented in other tissues by Staehelin and van Deurs and Luft.

Protein Tracer Experiments

The observations of the tracer experiments using horseradish peroxidase (HRP) as an extracellular marker in frog lenses are reported. The tracer studies were conducted in a "wash-out" procedure to compare with the conventional "nonwash" tracer studies. In the "nonwash" experiments, Figure 3 shows the presence of HRP reaction product in the lens after 15 min of incubation in the enzyme, within the eye. Reaction product is present in the lens capsule, intercellular spaces between epithelial cells, and between epithelium and fiber cells, and in the intercellular spaces of cortical fiber cells. We have also observed that within this time course (15 min), the depth of enzyme penetration into intercellular spaces of anterior cortical fibers is deeper in the equatorial and...
peripheral regions than in the very anterior central zone. This is in agreement with the findings of Gorthy et al. [3].

In the "wash-out" experiments, ten lenses were used and washed in TC-199 (70%) for various periods of time (2, 7.5, 10, 15, and 20 min) immediately following 15 min of the enzyme incubations, then fixed and processed for cytochemistry. Figures 3B and 4A-E show reaction product in the 10-min wash lens at the electron microscope level. Figure 3B shows that the reaction product is restricted to the apical end of the lateral intercellular spaces of the epithelial cells, and to the intercellular space between epithelium and fiber cells, and between cortical fiber cells. Reaction product in the capsule and most portions of the lateral epithelial intercellular spaces has been washed away (Figs. 3B, 4A-E). These results, which were obtained consistently, have also been confirmed in the lenses after 2, 7.5, 15, and 20 min of washing. A moving out of reaction product from the intercellular space between cortical fiber cells upon longer periods of washing (eg, 15 ~ 20 min) was noticed. Figures 4A-E show consecutive lateral intercellular spaces of epithelial cells with the tracer blocked at the same level.

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**Fig. 3.** A, TEM of a frog lens in a "nonwash" horseradish peroxidase (HRP) experiment (15-min enzyme incubation). Reaction product is localized in the capsule (C), epithelial intercellular spaces; intercellular spaces between epithelium and fiber cells, and between cortical fibers (F). Lead citrate stain. N = Nucleus. Bar = 1 μm; B, TEM of a frog lens in a "wash-out" HRP experiment (15-min enzyme incubation, 10-min wash). Reaction product is restricted to the locations apical to the zonulae occludentes near the apex of the epithelium (arrows), and to the intercellular spaces between epithelium and fiber cells, and between cortical fiber cells (F). Inset shows areas of membrane fusion (arrow heads) near the apex of the epithelial intercellular space. The reaction product does not appear basal to these areas of membrane fusion. Lead citrate stain. Bar = 1 μm. Inset, bar = 0.2 μm.
In our experience, when the section angle was favorable, areas of membrane fusion in the lateral intercellular spaces of the epithelial cells were always seen at the location where tracer was blocked (Fig. 3B, inset). Visualization of a greater number of membrane fusions was facilitated by the use of a goniometer stage.

Some reactive vesicles and dense bodies were found in the cytoplasm of epithelial cells in both wash and nonwash lenses (Figs. 3A, B and 4A–E).

**Discussion**

With the use of the “double mounting” method for making replicas of monolayer epithelial cells, the search and identification of the tight junctions (zonulae occludentes) become easier. To our knowledge this is the first time that the existence of zonulae occludentes has been demonstrated in the lens epithelia of human and frog.

There is a limitation of the freeze-fracture technique in the study of zonulae occludentes in the lens epithelium. Highly tortuous lateral membranes of these epithelial cells (Figs. 3A, B) make this technique extremely difficult to obtain large fracture faces of the lateral membranes. This may explain why zonulae occludentes are not found easily in the lens epithelium by using this technique. For the very same reason one might argue that a portion of the zonulae occludentes found in the lens epithelium may belong to the maculae occludentes, which do not show beltlike configuration. Therefore, the tracer experiments become important to clarify this question.

Our light and electron microscopic observations confirm that the tracer diffuses into the lens capsule, intercellular spaces between epithelial cells, between epithelium and fiber cells, and between cortical fiber cells from anterior, equatorial, and posterior regions in the (“nonwash”) lenses. This result is in agreement with the earlier findings of Gorthy et al. and Goodenough et al. There are three possible pathways for the tracer to diffuse into the intercellular spaces of the epithelial cells: (1) from anterior capsule to epithelium and underlying fibers, (2) from the intercellular space between epithelium and fibers (this originates from the intercellular space between the most...
equatorial epithelial cells and fibers) back to the epithelium and anterior capsule, and (3) from both directions described above and joining together in the intercellular space of the epithelial cells. The “wash-out” experiments clearly show that within various time intervals of washing the HRP reaction product is consistently restricted to the location of membrane fusion (zonulae occludentes), as seen with thin-section TEM. This corresponds to the location of the zonulae occludentes found in the freeze-fracture studies. These data strongly suggest that there are zonulae occludentes in the frog lens epithelium and that these structures do provide a barrier function for the transepithelial diffusion of the protein (HRP) with a molecular weight of 40,000 daltons.

It is possible that the zonulae occludentes in the lens epithelium may be “leaky” to low molecular weight substances (eg, Lanthanum, procion yellow) and “tight” to molecules with higher molecular weight. In this respect, we are planning to use various protein tracers with different molecular weights and ESR (Einstein-Stokes radius) in the permeability studies of lens epithelia in various species.

Key words: tight junction, zonulae occludentes, lens epithelium, human, frog, freeze-fracture, protein tracer HRP, electron microscopy

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