ATPase Pump Site Density in Human Dysfunctional Corneal Endothelium

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Proper corneal hydration is maintained by a Na, K-ATPase pump located in the lateral membranes of the endothelial cells. In dysfunctional corneas this pumping action appears to break down as the corneas become edematous. In order to provide quantitative and qualitative data on the Na, K-ATPase pump site density on dysfunctional and functional human corneal endothelial cells, the present study has employed both autoradiographic and histochemical techniques. Computer-assisted morphometrics and statistical analysis showed that there was a significant reduction \((P < 0.001)\) in \(^3\)H-ouabain binding, and thus ATPase pump sites, in the three types of corneas (Fuchs' endothelial dystrophy, aphakic and pseudophakic bullous keratopathy) with dysfunctional endothelia as compared to both types of corneas (eye bank, keratoconus) with functional endothelial cells. There were no significant differences amongst the dysfunctional types or between the two functional types of corneal endothelial cells in respect to density of silver grains. Histochemical staining for ATPase\(^1\) showed less p-nitrophenylphosphatase histochemical reaction product present on dysfunctional endothelial lateral membranes than in the functional cells. Invest Ophthalmol Vis Sci 28:1955-1962, 1987

Corneal clarity is maintained by the pump-leak mechanism of the corneal endothelium.\(^2\) While the precise pumping mechanism of this flux of bicarbonate and sodium ions is not fully known,\(^3\) it is believed to be influenced by a Na, K-ATPase pump located on the lateral intercellular membrane.\(^4-7\) Studies using the cardiac glycoside ouabain, a specific inhibitor of Na, K-ATPase,\(^8\) have shown that it inhibits the temperature reversal recovery of cold swollen corneas\(^9,10\) as well as causing a dose-related swelling in rabbit and human corneal endothelium.\(^11\) This evidence suggests that the pump function of the corneal endothelium is dependent on this Na, K-ATPase pump.

In dysfunctional corneas the normal balance between fluid leaking into the stroma and endothelial pumping appears to break down as these corneas become edematous. To date, studies investigating this deficiency in human dysfunctional corneal endothelium have suggested that the overall endothelial pump rate is not decreased in early Fuchs' endothelial dystrophy and may, in fact, be significantly increased over that of normal subjects\(^12\) due to the increased permeability of the corneal endothelium in Fuchs' endothelial dystrophy.\(^13-15\) In contrast, freeze-fracture data\(^16,17\) on end stage disease endothelia have demonstrated that there is a decrease in the number of intramembrane particles which may be related to a reduction in ATPase molecules in the lateral intercellular membranes of Fuchs' endothelial dystrophy cells and aphakic and pseudophakic bullous keratopathy endothelial cells.

In order to provide quantitative data on end stage corneal endothelial dysfunction, both a primary dystrophy (Fuchs' endothelial dystrophy) and surgically related dystrophies (aphakic and pseudophakic bullous keratopathy), the present study has employed autoradiographic methods in combination with computer-assisted morphometrics and statistical analysis. In addition, histochemical localization of the ATPase enzyme has provided data on the relative activity and location of this enzyme in these dystrophic conditions.

Materials and Methods

Tissue Collection

Experimental corneal buttons were obtained from eyes undergoing penetrating keratoplasty for Fuchs' endothelial dystrophy, aphakic bullous keratopathy and pseudophakic bullous keratopathy. Functional endothelium was obtained from keratoconus patients.
at the time of transplant surgery or from age-matched eye bank eyes obtained within 1 hr postmortem. All patients undergoing these procedures gave informed consent. Keratoconus eyes were examined preoperatively to ensure that there were no endothelial abnormalities or previous history of hydrops. At least three specimens from each corneal type were used.

**Autoradiography**

**Tissue preparation:** Corneal buttons were placed in McCarey-Kaufman (M-K) media immediately after surgical dissection and transported to the laboratory where incubation began within 15 min of removal from the patient. The preparation of the tissue for autoradiography is based on procedures modified from the methods of others. Briefly, the corneal buttons were divided in half, with the half which served as a control being preincubated with $10^{-6}$ M non-radioactive (cold) ouabain (ICN Pharmaceuticals, Plainview, NY) in M-K media for 15 min. The preincubation of the tissue with the non-radioactive ouabain provided competitive inhibition for the non-covalent binding of the steroid nucleus of the radioactive ouabain with the receptor. The second half remained in M-K media for 15 min and was then transferred to $7 \times 10^{-7}$ M $^3$H-ouabain (New England Nuclear, Boston, MA: specific activity 20 Ci/mMol) for 60 min. The radioactive media for control tissue continued to contain $10^{-6}$ M cold ouabain. The tissue was rinsed extensively in 0.1 M sodium cacodylate buffer (Polysciences, Warrington, PA), cut into squares, placed on aluminum foil supports, frozen in liquid Freon and stored in liquid nitrogen. The tissue was subsequently freeze-dried in a Balzer’s BAF-301 freeze-fracturing apparatus (Balzer’s Union, Hudson, NH) under a $10^{-7}$ torr vacuum. Once dried, the tissue was osmicated with vapors under vacuum for 12 hr to establish the tissue and the developed silver grain density was determined using the alternating grid square method over areas which contained no tissue.

For sampling, a cascade design was employed with a minimum of three corneas per condition being studied. The statistical analysis that was used in the present study takes into account that biological variation is usually the largest source of variation and also accommodates the unequal sample sizes that are commonly encountered in biological investigations. Statistical comparisons were subsequently made between the experimental and control tissue for each corneal button as well as between the different categories of functional and dysfunctional endothelia. Silver grain development in the basal epithelial layer of the corneas served as an internal confirmation of the viability of the techniques.

In order to examine a possible reduction in pump site density induced by the increased cell size of dysfunctional cells, silver grain density was also calculated on a per cell profile basis by counting the number of silver grains within each cellular cross sectional area.

**Histochemistry**

Corneal buttons in M-K media were slowly infused over 15 min at room temperature with a solution containing 3% paraformaldehyde (EMS, Fort Washington, PA), 0.25% glutaraldehyde (Ladd, Burlington, VT) in a 0.1 M sodium cacodylate (Polysciences) buffer with 5% sucrose (Sigma, St. Louis, MO). The corneal button was then transferred to full strength 4% paraformaldehyde and 0.25% glutaraldehyde for 1 hr. Following fixation the tissue was rinsed first in a 5% cacodylate buffered sucrose solution and then a 5% tris-buffered sucrose solution before being cut into 1 mm pieces. Histochemical localization of ATPase activity was accomplished using the p-nitrophenylphosphate (NPP) technique of Ernst. Control tissue was incubated in either ouabain, which binds to the ATPase, molecules, or without the NPP substrate. Following incubation the pieces of tissue were osmicated, dehy...
Fig. 1. Brightfield light micrograph showing the freeze-dried endothelium (arrow heads) of an age-matched eye bank eye cornea. The endothelial cells from keratoconus corneas appeared morphologically identical to eye bank eye endothelial cells. S = stroma. Bar = 20 μm.

Fig. 2. Darkfield light micrograph showing the silver grain density over the endothelium from a keratoconus cornea. Silver grains (arrow heads) appear dense in both types of functional endothelial cells (eye bank and keratoconus). S = stroma. Bar = 20 μm.

Fig. 3. Darkfield light micrograph showing the silver grain development in the control half of the cornea illustrated in Figure 2. Silver grain density in the endothelium (arrows) of control tissue was drastically reduced in comparison to experimental tissue. S = stroma. Bar = 20 μm.

Results

Functional Endothelium

When functional (keratoconus, eye bank) corneas were incubated in ³H-ouabain, the endothelial cells and the basal cells of the epithelium bound ³H-ouabain. The endothelial cells from both types of corneas appeared morphologically identical in light microscope sections (Fig. 1) as well as being heavily labelled with silver grains (Fig. 2). Grain density was not significantly different (P = 0.2) between keratoconus and eye bank eyes. The functional endothelium had an average of 84.67 silver grains per 100 μm² of endothelium which was significantly different (P < 0.001) from background and control tissue (Fig. 3) (see below).

Dysfunctional Endothelium

Dysfunctional (Fuchs' endothelial dystrophy, aphalic (ABK) and pseudophakic (PBK) bullous keratopathy) corneas were similar to the corneas with functional endothelium in that both the endothelium and the basal cells of the epithelium bound ³H-ouabain. In contrast to the functional cells, the endothelia from all three types of dysfunctional corneas (Fig. 4) showed very few silver grains (x = 17.37 silver grains/100 μm²). This silver grain density was significantly different from background and control tissue (Fig. 3) (see below).
Fig. 4. Darkfield light micrograph showing the endothelium from a pseudophakic bullous keratopathy cornea as an example of a dysfunctional endothelium. The silver grain (arrow heads) density is reduced in comparison to the density over functional endothelia. Folds (arrows) in the tissue section appear as bright lines when viewed by darkfield microscopy. $S =$ stroma. Bar = 20 $\mu m$.

Fig. 6. Darkfield light micrograph of the epithelium from a dysfunctional cornea (ABK) showing silver grain development in the basal cell layers. This silver grain (arrows) development showed the validity of the techniques when there was reduced silver grain numbers in dysfunctional endothelia. $S =$ Stroma. Bar = 20 $\mu m$.

Fig. 5. Histograms comparing the silver grain density values obtained from: 1) eye bank eye; 2) keratoconus; 3) Fuchs' endothelial dystrophy; 4) aphakic bullous keratopathy; and 5) pseudophakic bullous keratopathy corneas. The mean values ± SEM are illustrated. No statistical differences were observed amongst the groups (functional; 1 and 2 or dysfunctional; 3, 4 and 5). However, dysfunctional endothelial had significantly ($P < 0.001$) fewer silver grains per 100 $\mu m^2$ as compared to functional endothelia.

Fig. 2. The human corneal endothelium $^{3}H$-ouabain binding.

Controls

Control tissue for both functional (Fig. 3) and dysfunctional endothelia were incubated in non-radioactive ouabain in addition to $^{3}H$-ouabain. Endothelial cells showed silver grain development no higher than the background levels indicating that binding of the radioactive ligand in experimental tissue was specific.

An internal confirmation of the techniques was evident in experimental tissue which showed silver grain development over the basal cell layers of the corneal epithelium (Fig. 6). This development, which was significantly increased in comparison to the control tissue, indicat
Fig. 7. Transmission electron micrographs showing electron dense ATPase histochemical reaction product (arrows) along the lateral membranes of corneal endothelial cells. The functional cell type (A—keratoconus) has qualitatively more reaction product than the dysfunctional cell type (B—Fuchs' endothelial dystrophy). D = Descemet's membrane; PCL = posterior collagenous layer. Bar = 1 μm.
background, showed the validity of the techniques. This was especially important in dysfunctional corneas where the density of the \(^3\)H-ouabain was very low over the endothelium, but remained high over the basal epithelial cells.

**Histochemical Localization**

Qualitative transmission electron microscopic observations supported the autoradiographic results. Functional endothelia from both eye bank and keratoconus corneas showed electron-dense reaction product along their lateral membranes (Fig. 7A). Endothelial cells from all three types of dysfunctional corneas (Fuchs', ABK, PBK) had reaction product scattered along their intercellular membranes in a patchy distribution that appeared less dense than that of the functional endothelia (Fig. 7B).

Control tissue confirmed the specificity of the histochemical localization. Both the ouabain inhibition of binding and the control tissue that was incubated without the substrate showed no reaction product along the lateral cell borders.

**Discussion**

Ouabain has previously\(^{32}\) been shown to bind in a 1:1 ratio to Na, K-ATPase molecules. This binding property has previously been used\(^{19-21}\) to show by autoradiographic techniques that ATPase pump sites are located in the basal lateral membranes of transporting epithelia. In addition to showing ATPase activity in the basal intercellular borders of the corneal epithelium, the present study has shown that there is both a quantitative as well as a qualitative difference in ATPase pump site density between functional and dysfunctional corneal endothelium. Dysfunctional endothelium from three pathological corneal conditions (Fuchs', ABK, PBK) showed a four-fold quantitative reduction in the amount of \(^3\)H-ouabain bound to their membranes and thus a reduction in the number of ATPase pump sites. In addition, histochemical localization of the ATPase enzyme showed a qualitative decrease in ATPase enzyme activity in comparison to the functional endothelial cells.

Comparisons between both types of functional corneas and the dysfunctional corneal endothelial cells could not be made until keratoconus and eye bank eye endothelial cells were judged regarding their similarity. Previous freeze-fracture\(^{16,17}\) and freeze-fracture label studies\(^{33}\) have shown that morphologically, there are no differences between keratoconus corneal endothelial cells and corneal endothelial cells from eye bank eyes age matched with dysfunctional endothelial specimens. In addition, results from the present study indicate that there is no statistically significant difference in ATPase pump site density between these two corneal endothelial cell types.

The reasoning behind using keratoconus corneas as functional tissue in this study was two-fold. First, since these studies were concerned with enzyme activity, it was concluded that only corneas from eye bank eyes obtained less than 1 hr postmortem would be acceptable for study in order to minimize postmortem changes. Corneal buttons from keratoconus patients undergoing penetrating keratoplasty are more readily available than acceptable eye bank corneas and have not been subjected to any deleterious postmortem changes. Second, the three types of dysfunctional endothelial tissue were being processed after being surgically removed. Keratoconus corneal buttons were also removed surgically, thereby providing identical experimental conditions. The inclusion of keratoconus corneal endothelium as a functional corneal endothelium in this and other studies\(^{16}\) appears to be valid based on clinical, morphological and autoradiographic observations of the endothelium as well as the fact that the defect in the condition of keratoconus is believed to occur in the basal epithelial cells, their basement membrane and Bowman's layer.\(^{34}\)

When Fuchs' endothelial dystrophy cells were compared to the functional endothelial cells of both age-matched eye bank eye and keratoconus corneas, they showed a dramatic quantitative decrease in ATPase pump site density. Previous reports\(^{12}\) suggest that the endothelial pump site density is not decreased and may in fact be increased in comparison to functional corneas in response to the increased permeability of the corneal endothelium in Fuchs' endothelial dystrophy.\(^{13-15}\) The possible explanation for these conflicting results may be inherent in the relative severity of the disease in the subject corneas. The previous study\(^{12}\) used corneas in a relatively early stage of Fuchs' dystrophy while the present study used subjects in the end stage of the disease when the cornea has decompensated. The possibility exists that in the early stages an increase in pump site density exists, but in the final stage of the disease process a dramatic decrease in the pump site density occurs. The resolution of whether this dramatic decrease occurs or whether there is a more gradual decrease in pump site density requires a quantitative comparison made throughout the entire progression of the disease.

The autoradiographic results for the two types of cataract-related endothelial disorders are similar to those obtained for Fuchs' endothelial dystrophy corneas. Aphakic and pseudophakic bullous keratopathy
corneal endothelial cells showed a greatly reduced ATPase pump site density in comparison to the pump density on functional endothelial cells. Although the edema noted in these conditions may be partially related to a decrease in endothelial cell density, our results indicate that there is not a compensatory increase in pump site density on the remaining cells. Corneal edema in these conditions may be ultimately due to a variety of factors; however, it would appear from these data that the decrease in pump site density should be considered one of the major factors contributing to the corneal edema in these disorders.

In order to examine a possible reduction in pump site density induced by the increased surface area of dysfunctional cells, silver grain density was also calculated on a per cell profile basis. These data were compared to a hypothetical model which is based on equal numbers of pump sites on both functional and dysfunctional cells. This comparison revealed that the hypothetical model showed only a slight difference in the ratio of pump sites on functional to dysfunctional cells (5:3), while our data showed a very large ratio (8:1). The magnitude of this ratio suggests that the decrease in pump site density observed on dysfunctional cells is not entirely due to a dispersion of the pump sites over the larger membrane area, but rather a reduction in the total number.

Qualitative histochemical observations on the corneal types used in the present study have supported the quantitative data. Similar to results of previous studies, the ATPase reaction product was localized to the intercellular membranes of both functional and dysfunctional corneal endothelial cells. Dysfunctional endothelia had less histochemical reaction product and thus less ATPase activity along their lateral cell borders than did their functional counterparts. While this ultrastructural qualitative data by itself cannot be used to distinguish between functional states, this data does support the observations made here on light microscope autoradiographs.

In conclusion, the present study has provided both quantitative and qualitative data that show that human dysfunctional corneal endothelial cells have decreased ATPase pump site density as compared to functional cells. We suggest that this decrease in pump site density may be a contributing factor to the corneal edema that is present in these end-stage endothelial dystrophies. The underlying cellular factors behind this decrease remain to be elucidated in future studies.

Key words: ATPase, autoradiography, corneal endothelium, dysfunctional, human

References

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