Freeze Fracture Study of Human Corneal Endothelial Dysfunction

Yuko Sasaki, Audrey W. Tuberville, Thomas O. Wood, and Barbara J. McLaughlin

Intramembrane changes occurring in dysfunctional corneal endothelial cell membranes were examined using freeze-fracture and transmission electron microscopy techniques. Three categories of dysfunctional endothelium were examined: aphakic bullous keratopathy, pseudophakic bullous keratopathy and Fuchs' endothelial dystrophy. Keratoconus corneas and a donor eye bank eye were examined as normal controls. Four intramembrane changes were observed on replicas of freeze-fractured membranes in each category of dysfunctional endothelium. These were a marked reduction in intramembrane particle density on lateral membranes, altered apico-lateral junctional complexes, increased vesicle fusion sites on apical, lateral and basal membranes, and abnormal desmosome-like particle aggregates on the lateral endothelial cell membranes. The marked reduction in intramembrane particles on lateral membranes may be due to a change in the macromolecular components associated with pump dysfunction. The increase in membrane vesicle fusion sites and the breakdown in intercellular junctions may be associated with increased permeability during barrier dysfunction. Invest Ophthalmol Vis Sci 27:480-485, 1986

There are a number of clinical entities with associated irreversible corneal edema in which pump and/or barrier dysfunction play a role: congenital abnormalities such as congenital hereditary endothelial dystrophy (CHED); endothelial dystrophic processes such as Fuchs' endothelial dystrophy (FED); and/or surgical trauma leading to bullous keratopathy, either aphakic (ABK) or pseudophakic (PBK). In these disorders, altered corneal endothelial barrier or pump function can upset the equilibrium forces with ensuing corneal edema. Chronic stromal edema results in the collection of water under areas of the corneal epithelium causing elevations called bullae, a condition known as bullous keratopathy. Since edema greatly reduces corneal transparency, all of the above entities require corneal transplantation for visual rehabilitation.

The exact cellular mechanisms of endothelial pump and barrier dysfunction are unknown. The objective of this study was to examine for the first time intramembrane changes occurring in dysfunctional endothelial membranes using freeze-fracture and correlative transmission electron microscopy.

Materials and Methods

Tissue Collection

Experimental corneal buttons were obtained from 15 patients in three dysfunctional endothelial diagnostic categories at the time of corneal transplant surgery. There were six ABK, four PBK, and five FED sample corneal buttons collected.

Four control buttons were obtained, three from keratoconus patients at the time of surgery, and one donor eye bank eye obtained within 2 hr postmortem. Keratoconus eyes were used for control tissue only when there was no evidence of endothelial abnormalities as determined by transmission electron microscopy. All corneas were fixed and prepared for freeze fracture and transmission electron microscopic study according to the following methods.

Freeze Fracture

Corneal buttons were placed in McCarey–Kaufman (M–K) media immediately after surgical dissection and fixed by slow addition of an equal volume of fixative to the M–K media during a 15-min period. The fixative contained 1% paraformaldehyde, 1% glutaraldehyde, 0.5% acrolein in 0.1 M sodium cacodylate buffer at pH 7.3. The tissue was then transferred to full strength fixative and dissected into two halves. The half prepared for freeze-fracture was fixed for 2 hr in full strength fixative. The tissue was then washed in 0.1 M caco-
dylate buffer, pH 7.3, equilibrated with 23% (v/v) glycerol–water for 2 hr, hand cut into squares, and mounted with epithelium-side placed down on the gold specimen holder. The mounted tissue was frozen in liquid nitrogen, cooled Freon 22 and stored in liquid nitrogen. The endothelial tissue was fractured at −116°C in the plane of the endothelium at different levels and replicated in a Balzers BAF 301 freeze-fracture apparatus fitted with an electron beam gun for platinum shadowing and a quartz crystal monitor for standardizing replica thickness. The replicas were cleaned in methanol and Purex and mounted on Formvar-coated grids. Replicas were examined and photographed with a Philips 301 electron microscope (Eindhoven, The Netherlands).

Transmission Electron Microscopy

The other half of corneas corresponding to those fixed for freeze fracture were fixed overnight with the same fixative used for the freeze fracture study. Tissue was postfixed with 2% osmium tetroxide, stained en bloc with 2% uranyl acetate, dehydrated in a graded series of ethanols, and embedded in Epon/Araldite. Thin sections were cut on an ultramicrotome, stained with uranyl acetate and lead citrate, and examined in the electron microscope.

Results

Freeze Fracture Studies

Corneas were mounted with epithelium placed down on specimen holders so that the fractured planes obtained were P-faces (protoplasmic leaflets) of the apical cell membrane, P-faces and E-faces (external leaflets) of lateral cell membranes, and E-faces of basal cell membranes.

Control Endothelium—Eye Bank Eyes and Keratoconus Eyes

No differences between keratoconus and donor eyes were observed.

The apical endothelial cell membranes of control corneal endothelium were characterized by closely packed, evenly distributed intramembrane particles and occasional vesicle fusion sites (Fig. 1).

Lateral cell membranes were characterized by dense, evenly distributed particles on the P-face membranes and sparse particles on the E-face lateral membrane leaflets (Fig. 2). Two types of junctions were observed on the lateral membranes. Apico-lateral junctional complexes were present at the apical margins of the endothelial cells, consisting of tight junctional strands and gap junctional particle aggregates enclosed within or adjacent to the tight junctional strands (Fig. 3). The tight junctional strands appeared as an anastomotic meshwork of particle rows within grooves on the E-face membrane and corresponding patterns of ridges on the P-face membrane leaflets. Isolated gap junctional particle aggregates were also seen along the remainder of the lateral membrane away from the apical border (Fig. 2). They consisted of densely packed, hexagonal particle aggregates of various sizes, which were either hexagonal or round in shape. No other junctional

---

Fig. 1. Freeze-fracture of normal apical endothelial membrane from eye bank eye showing two adjacent cells joined at their apico-lateral borders (arrows). The intramembrane P-face (Pf) particle density is high and some vesicle fusion sites are present (arrowheads) (×44,000).

Fig. 2. Freeze-fracture of normal lateral endothelial membrane from keratoconus eye showing high intramembrane particle density and gap junctional particle aggregates (arrowheads) on the P-face membrane (Pf). Particles are characteristically sparse on adjacent E-face (Ef) membrane of an adjoining cell (×62,500).
structures were observed on lateral endothelial membranes.

Basal E-face membranes of control endothelium were characterized by evenly distributed particles of low density. The interdigitating, cross-fractured intercellular spaces surrounding each basal cell were also seen, as well as occasional vesicle fusion sites in the basal membrane (Fig. 4).

**Dysfunctional Endothelium**

No substantial differences were observed between membrane structures in the three dysfunctional categories (ABK, PBK, FED).

Apical cell membranes of dysfunctional endothelial cells were characterized by numerous vesicle fusion sites (Fig. 5) and in most of the pathologic cases, the packing density of intra-membrane particles appeared to be less than in the normal control corneas.

On the lateral membranes, intramembrane particle densities appeared to be markedly reduced in most pathologic specimens that were examined (Figs. 6, 9). Vesicle fusion sites were increased (Fig. 6) as compared to normal lateral membranes from keratoconus or eye bank eyes. In four cases (1 PBK, 1 ABK, 2 FED), no tight junctions were observed on apico-lateral membranes. In other cases some tight junctional strands with smaller gap junctional arrays were observed (Fig. 7). They were, however, less extensive than those found in keratoconus and eye bank eyes and were frequently open-ended and disrupted with few anastomosing strands. In the remainder of the dysfunctional corneas, only isolated tight junctional fragments or disorganized junctional strands were present on widely exposed lateral membranes (Fig. 8).

An unusual particle aggregate was observed on apico-lateral membranes in one case out of each dysfunctional category. These structures consisted of small clusters of P-face intramembrane particles resembling desmosomal aggregates (Fig. 9). These particle aggregates, which were also observed on E-face membranes (see Fig. 9, inset) were loosely packed, and of uneven size. In corresponding thin sections from these same cases, desmosome-like junctions were present between the apical endothelial cell borders (Fig. 9, inset).
Fig. 6. Freeze-fracture of overlapping lateral membranes from an eye with Fuchs' endothelial dystrophy. The P-face (Pf) membrane leaflet of one cell has numerous vesicle fusion sites (arrowheads). Adjacent membrane E-face (Ef) leaflets resemble the P-face membranes of an underlying endothelial cell in that the particle density and distribution is similar (X39,900).

Fig. 7. Freeze-fracture of apico-lateral cell junctions from eye with ABK. The tight junctional strands (arrows) are fewer in number and more disrupted than those seen in normal eyes and the gap junctions (arrowheads) are smaller and appear fragmented (X98,800).

Fig. 8. Freeze-fracture of apico-lateral cell junctions from eye with PBK showing isolated fragments of a tight junctional strand (arrows). Vesicle fusion sites are present in the adjacent membrane area (arrowheads) (X62,700).

Discussion

Four pathologic changes were observed on replicas of freeze fractured membranes of corneas with dysfunctional endothelium. One abnormal feature was a reduction in intramembrane particles on lateral membranes. Secondly, altered apico-lateral junctional complexes were observed. Thirdly, there were increased vesicle fusion sites on apical, lateral and basal membranes and fourthly, unusual desmosome-like junctions were present on the lateral endothelial cell borders.

Intramembrane particles seen in freeze-fracture replicas are thought to represent transmembrane proteins. Some intramembrane particles are related to the presence of transport proteins, such as Na⁺/K⁺ ATPase. Investigators have demonstrated in purified fractions of Na⁺/K⁺ ATPase enriched membranes from kidney and in membranes reconstituted with purified Na⁺/K⁺ ATPase, that the enzyme protein can exist as a freeze-fractured particle. In the cornea, ATPase-dependent Na⁺/K⁺ ion pump sites have been shown to be present on lateral membranes of endothelial cells. It is possible that some of the intramembrane particles observed here in corneal lateral, endothelial cell membranes may represent ATPase ion pump sites. In this

Transmission EM Studies

Control endothelium: Thin sections of endothelium of control corneas from both eye bank eyes and keratoconus eyes demonstrated continuous, cuboidal, monolayered cells with interdigitated borders. Normal apico-lateral tight and gap junctional complexes were observed in both keratoconus and eye bank eyes and occasional vesicle fusion sites were observed on either basal or apical membranes.

Dysfunctional endothelium: Thin sections of dysfunctional corneas revealed flattened endothelial cells which overlapped each other and formed multiple layers. Abnormal desmosome-like junctions were observed between apicolateral and lateral junctional complexes along the overlapping areas of cell membranes (Fig. 9, inset). Tight and gap junctions were usually not present. The number of vesicle fusion sites on both apical and basal cell membranes and intracyttoplasmic vesicles appeared to be increased (Fig. 11). These pathological changes were observed in all three pathologic categories.

Basal membranes of the dysfunctional endothelium group were characterized by increased vesicle fusion sites on the basal E-face membranes (Fig. 10). Intercellular borders were not observed as often as in normal endothelium since the cells were greatly enlarged and flattened. The sparse background particle density appeared similar to normal E-face basal membranes from keratoconus or eye bank eyes.
regard, the decreased particle density observed in our study, particularly on lateral membranes, may indicate that the number of pump sites is decreased in dysfunctional endothelial cells. Preliminary quantitative results in this laboratory indicate that there is a 25% reduction in particle density on the lateral endothelial membranes from all three pathologic specimens as compared to normal controls.

In conflict with the observation above is the study by Geroski et al. using tritiated ouabain, which showed that ATPase pump site density was significantly increased in endothelium of a postmortem human eye with Fuchs' dystrophy. This discrepancy may be a function of sampling at differing points in the time course of the natural history of increasingly dysfunctional endothelium. The corneas examined for this study were from eyes transplanted for severely dysfunctional endothelium with chronic irreversible stromal edema. At this late stage of dysfunctional endothelial disease, the pump sites may well be decreased in number.

Normal intact intercellular junctions between corneal endothelial cells have been reported previously in freeze-fracture and ultrastructural studies. In the three categories of dysfunctional endothelium studied here, various degrees of junctional alteration from the normal pattern were observed. Inamotod and DeVoe showed that in Fuchs' dystrophy corneas, tight junctions were often shorter or not present. Burns et al. demonstrated in Fuchs' dystrophy patients with moderate disease, that endothelial permeability to fluorescein was significantly increased whereas the endothelial pump rate was normal. They concluded that the earliest defect in Fuchs' dystrophy was solely a breakdown in barrier function. The severely altered junctional complexes observed in this study which subserve barrier function supports Burns' et al. conclusion that Fuchs' dystrophic endothelium has a decreased ability to exclude water from the stroma. In all of our specimens from patients with endothelial dysfunction, we found a decreased intramembrane particle density, as well as junctional derangement, suggestive of membrane alterations that may be related to a decrease in ion pump sites and barrier dysfunction. It is possible that the normal complement of ion pump sites pump at a greater rate in the early and moderate stages of dysfunctional endothelial states when barrier dysfunction predomi-
nates. It seems likely that in the later stages of dys-
function, as pump sites become decreased in number, 
the maximum pump rate will be reached. As ion pump 
sites become further decreased, the cell will no longer 
be able to maintain stromal water equilibrium and ir-
reversible stromal edema will ensue. Indeed, we ob-
served membrane changes in all the late stage speci-
mens examined that may be associated with pump and 
barrier dysfunction.

Tanaka, et al\textsuperscript{18} showed numerous vesicle fusion sites in 
the corneal epithelium of rabbit and Rhesus monkey 
by freeze-fracture techniques which they suggested were 
nutrient transport vesicles. In our study numerous ves-
icle fusion sites on apical, lateral and basal membranes 
of dystrophic endothelium were observed that may be 
indicative of increased permeability or transcellular 
fluid transport of dysfunctional endothelial cells. Future 
studies with extracellular tracers will determine whether 
the net movement of these vesicles is endocytotic or 
exocytotic.

Specific particle aggregates not seen in normal cor-
neal endothelium were observed in three pathologic 
specimens, one in each diagnostic category. The ag-
gregates were located at lateral membrane regions on 
both P- and E-faces and had an appearance identical 
to that of freeze-fractured, desmosomal particle aggre-
gates. In corresponding thin sections from the same 
specimens, desmosome-like junctions were observed in 
similar membrane areas. Several investigators have 
previously reported fibroblast or epithelial-like changes 
of Fuchs' dystrophy endothelium,\textsuperscript{16,19,20} and abnormal 
desmosomal junctions have been observed between 
endothelial cells. These junctions, usually seen in tis-
ues of other embryonic origin, illustrate the pluripo-
tential nature of pathologic corneal endothelium.

In summary, the marked reduction in intramem-
brane particles on lateral membranes may be due to a 
change in the macromolecular components associated 
with pump dysfunction. The increase in membrane 
vessel fusion sites and the breakdown in intercellular 
junctions may be associated with increased perme-
ability reported in dysfunctional endothelium during 
barrier dysfunction. Future work is underway in this 
laboratory to correlate these freeze-fracture changes 
with functional components of corneal membranes 
during endothelial dysfunction.

Key words: freeze-fracture, Fuchs' endothelial dystrophy, 
aphakic bullous keratopathy, pseudophakic bullous keratop-
athy, corneal endothelial dysfunction

Acknowledgment

The technical assistance of Ms. Lou Boykins is gratefully 
acknowledged.

References

1. Waring GO, Bourne WM, Edelhauser HF, and Kenyon KR: 
The corneal endothelium. Normal and pathologic structure and 
2. Pinto da Silva P and Branton D: Membrane splitting in freeze-
etching. Covalently bound ferritin as a membrane marker. J Cell 
3. Yu J and Branton D: Reconstitution of intramembrane particles 
in recombinants of cytochrome protein Band 3 and lipid: effects 
of spectrin-actin association. Proc Natl Acad Sci USA 73:3891, 
1976.
4. Hong K and Hubbell WL: Preparation and properties of phos-
pholipid bilayers containing rhodopsin. Proc Natl Acad Sci USA 
5. Duguchi N, Jorgenson PL, and Maunsbach AB: Ultrastructure 
of the sodium pump. Comparison of thin sectioning, negative 
staining, and freeze-fracture of purified, membrane-bound 
6. Skriver E, Maunsbach AB, and Jorgenson PL: Ultrastructure of 
Na,K-transport vesicles reconstituted with purified renal Na,K-
7. Van Winkle WB, Laue LK, and Schwartz A: The subunit fine 
structure of isolated, purified Na\textsuperscript{+},K\textsuperscript{+}-adenosine triphosphatase. 
8. Kaye GI and Tice LW: Studies on the cornea. V. Electron mi-
croscopic localization of adenosine triphosphatase activity in 
the rabbit cornea in relation to transport. Invest Ophthalmol 5:22,
1966.
9. Leuenberger PM and Novikoff AB: Localization of transport 
10. Tervo T, Palva M, and Palkama A: Transport adenosine tri-
phosphatase activity in the rat cornea. A comparative histo-
11. Geroki O, Matsuda M, Yee RN, and Edelhauser HF: Quanti-
tation of Na/K ATPase pump site density in the human corneal 
of intercellular spaces and junctions in regenerating rabbit corneal 
structure of rabbit corneal endothelium by the freeze-fracture 
15. Ottersen O and Vegge T: Ultrastructure and distribution of in-
tercellular junctions in corneal endothelium. Acta Ophthalmol 
16. Iwamoto T and DeVoie AG: Electron microscopic studies on 
17. Burns KP, Bourne WM, and Brubaker RF: Endothelial function 
in patients with cornea guttata. Invest Ophthalmol Vis Sci 20:
77, 1981.
18. Tanaka M, Ohnishi Y, and Kuwabara T: Membrane structure 
of cornel epithelium: freeze-fracture observation. Jpn J 
scopic studies of corneal endothelium: the abnormal endothelium 
associated with retrocorneal membrane. Ann Ophthalmol 4:564, 
1972.