Electron microscopic changes in Descemet's Membrane(DM) in Pseudophakic bullous keratopathy and Fuchs endothelial dystrophy.

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Abstract:

Purpose: To elucidate ultra-structural changes of the DM and endothelial cells in Fuch's Endothelial Corneal Dystrophy (FECD) and Pseudophakic Bullous Keratoplasty(PKB) by scanning electron microscopy.

Methods: Descemet's membrane from 8 patients were collected following DSEK performed for Fuch's Endothelial Dystrophy in 3 patients and Pseudophakic Bullous Keratopathy in 5 patients. Thin sections were prepared which were then studied by Transmission Electron Microscopy.

Results: In FECD the DM was thickened with excrescence in all 3 cases and composed of 4 layers, though these 4 layers were not present in one FECD specimen. In FECD stripping specimen, we observed that the endothelium was markedly attenuated, especially over the excrescences, to atrophic in our 6 specimens. Excrescences are continuous with the third layer. Endothelial cells were completely degenerated in 2 specimens of PBK. In FECD, HCEC were thinned out, almost unnoticeable over the nodules. No nodules seen in PBK.

Conclusion: Fibroblast like morphological changes occur in endothelial cells in both FECD and PBK with increased rough endoplasmic reticulum, cytoplasmic filaments, lysosomes and increased degenerative changes like swollen mitochondria, however these changes are more marked in FECD than in PBK. DM shows abnormal posterior banded layer consistently in FECD but can also be found in PBK. DM thickening is more marked in FECD than in PBK. Features that are seen in FECD stroma are presence of lipid keratopathy, melanin granules separating it from PBK.

INTRODUCTION

Descemet's membrane is the basement membrane of the corneal endothelium. Unlike Bowman's membrane, it is a true basement and is continuously deposited throughout life by the underlying endothelium, becoming gradually thicker with age.At birth, it is only 2-4μ thick, but by adulthood it grows to 10-12μm. Ultra structurally, it can be divided into 2 zones: an anterior fetal banded zone and a posterior non-banded zone. The anterior banded zone is laid down by the endothelial cells only in embryogenesis and remains unchanged through life. The banded fibres are largely composed of type VIII collagen, a collagen commonly found in fetal tissues, especially around blood vessels. The growth in thickness later in life occurs primarily in the posterior banded zone.[1-4]

Irregularities and excrescences are common in the peripheral aspects of Descemet's membrane and are

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seen frequently with advancing age. These are referred to as Hassal-Henle warts. They do not interfere with vision and are not considered pathologic. When present in the central cornea, they are called guttae. Fuch's Dystrophy is the most common corneal dystrophy causing vision loss.[5,6]

The endothelium is composed of hexagonal endothelial cells.The Endothelial Cell Density (ECD) decreases throughout life. From birth to 14 years, the rate of endothelial cell loss is approximately 3% per year. After age of 14, the rate slows to about 0.6% per year. Specular microscopy in normal young adults corneas reveal an ECD of about 3500[7,8,9] cells/mm^2. The ECD declines to about 2000 cells/mm^2 in older age. As ECD decreases, individual cells enlarge and lose their hexagonal shape.[10,11] The critical ECD below which the cornea decompensates is approximately 300-500[12,13] cells/mm^2.

The endothelium forms the anterior border of the anterior chamber and is therefore susceptible to blunt or penetrating trauma such as cataract extraction or anterior chamber IOL implantation, e.g, endothelial cells damage leading to pseudophakic bullous keratopathy. Endothelial Dysfunction caused by pseudophakic Bullous Keratoplasty (PBK) and Fuch's Endothelial Dystrophy is the leading cause of corneal visual loss and leading indication for corneal transplantation.

Transmission Electron Microscope is recent and advanced tool for the assessment of ultrastructure of Descemet's membrane and endothelial cells.

AIM:

To elucidate ultra-structural changes of the DM and endothelial cells in Fuch's Endothelial Corneal Dystrophy (FECD) and Pseudophakic Bullous Keratoplasty(PKB) by scanning electron microscopy.

METHOD

The study was conducted in the Department of Ophthalmology, Institute of Medical Sciences, Banaras Hindu University, in collaboration with the Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University.

In our study, 8 patients, aged 45 to 65, undergoing DSEK (3 FECD cases and 5 PBK cases) consented to the use of their excised DM for evaluation under TEM.

In DSEK, the diseased DM were peeled off, fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 12 hours at 4^0 C. After wash in buffer, the samples were postfixed in 1% OsO_4 for 1 hour at 4^0 C. Ultimately, thin sections are prepared which were then elucidated by Transmission Electron Microscopy. The patients included in this study were diagnosed withFuch's Endothelial Dystrophy and PBK.

Fixation of DM and endothelial cells for TEM

Small pieces of tissues were cut and the tissue samples fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 12 hours at 4^0 C. After wash in buffer, the samples were postfixed in 1% OsO_4 for 1 hour at 4^0 C. The samples were dehydrated in an ascending grade of acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK). Thick Sections (1 µm) were cut with an ultramicrotome, mounted on to glass slides, stained with aqueous toluidine blue and observed under a

light microscope for gross observation of the area and quality of the tissue fixation. For electron microscope examination, thin sections of grey-silver color interference (70-80 nm) were cut and mounted onto 300 mesh- copper grids. Sections were stained with alcoholic uranyl acetate and alkaline lead citrate, washed gently with distilled water and observed under a Morgagni 268D Transmission Electron Microscope (Fei Company, The Netherlands) at an operating voltage 80 KV. Images were digitally acquired by using a CCD camera (Megaview III, Fei Company) attached to the microscope.

The samples were made free of culture medium by washing/centrifugation (1000X, 5 min) in 0.1 M Phosphate Buffer (PB, pH 7.3). The supernatant was discarded. The pellet (after dispersing the cells) was fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in PB for 2-3 hour at room temperature then centrifuged in PB for 5 min to remove the fixative. The pellet was suspended in PB, again centrifuged and washed. The samples (pellet) were postfixed for 1 hr in 1% osmium tetroxide at 4 Deg C. Samples were then dehydrated in acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK). Thick Sections (1 µm) were cut with an ultramicrotome, mounted on to glass slides, stained with aqueous toluidine blue and observed under a light microscope for gross observation of the area and quality of the tissue fixation. For electron microscope examination, thin sections of grey-silver colour interference (70-80 nm) were cut and mounted onto 300 mesh- copper grids. Sections were stained with alcoholic uranyl acetate and alkaline lead citrate, washed gently with distilled water and observed under a Morgagni 268D Transmission Electron Microscope (Fei Company, The Netherlands) at an operating voltage 80 kV. Images were digitally acquired by using a CCD camera (Megaview III, Fei Company) attached to the microscope.

OBSERVATIONS AND RESULTS:

Among these 8 patients, 3 were diagnosed to have Fuch's Endothelial Corneal Dystrophy and the remaining 5 as having Pseudophakic Bullous Keratopathy.

Slit-lamp examination in FECD patients, preoperatively, revealed the presence of guttatae, Fig 1.

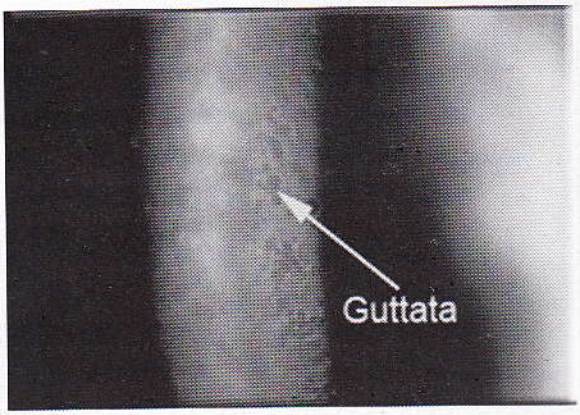
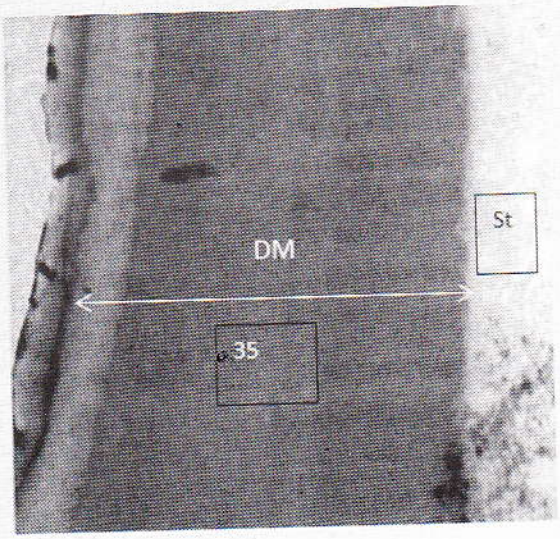


Fig 1. Slit-lamp direct illumination revealing presence of guttata on the posterior cornea in FECD

n slit lamp examination of bullous keratopathy stromal and epithelial edema and bullae were noted few weeks prior to DSEK, Fig 2.

Fig 2. Sclerotic scatter showing the presence of stromal edema and epithelial bullae



Stromal adherence was seen in 1 FECD stripping specimen, Fig 3.

Fig 3. Layers of DM in FECD with markedly attenuated endothelial cells . DM is 35µm thick. Stroma (St) is present in this DSEK stripping specimen X 3000

We observed that in FECD the DM was thickened with excrescences all 3 cases and composed of 4 layers, which are described morphologically, though these 4 layers were not present in one FECD specimen.

The first layer, anterior fetal banded layer, was present and relatively uniform in all FECD cases. Its thickness varied from 3-4µm. It was characterized by wide-spaced collagen. The second layer, posterior non-banded layer, was non-banded, homogeneous, less osmophilic than the first layer. Its thickness was approximately 4-5µm. The third layer, posterior banded layer, was banded and had an osmophilia as the first layer and its thickness was approximately 30µm. It consisted of spaced collagen. The fourth layer, fibrillar layer was approximately 7-9µm thick. It was composed of a loose matrix of collagen. Multiple waves of basal lamina were present in the fibrillar layer, Fig 4.

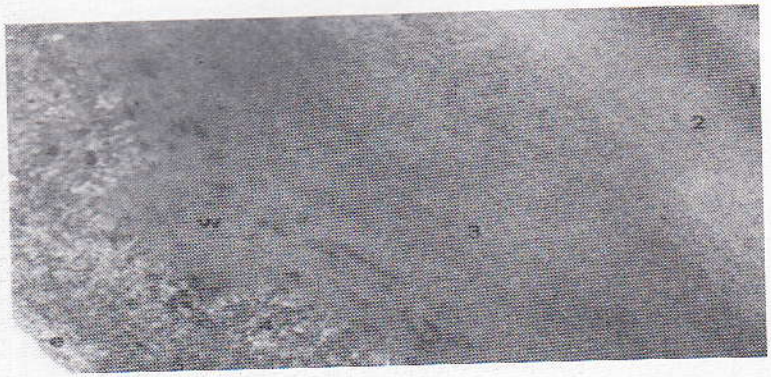


Fig 4. Descemet's membrane in FECD showing 4 layers: 1. Anterior fetal layer, 2. Posterior non-banded layer, 3. Posterior banded layer and 4. Fibrillar layer. W-Wart, E-endothelium X 5000

In FECD stripping specimen, we observed that the endothelium was markedly attenuated, especially over the excrescences, Fig 5.

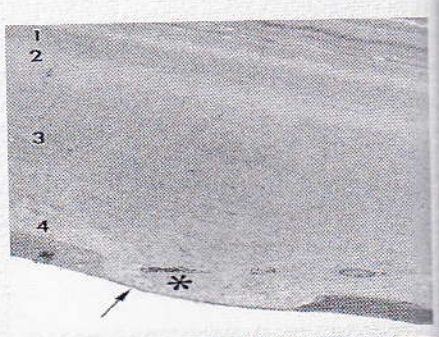
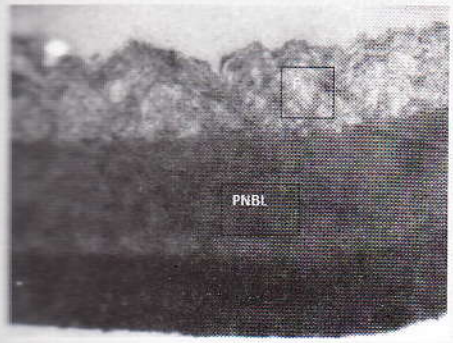


Fig 5. FECD: showing 4 layers, 1. Anterior fetal layer, 2.Posterior non-banded layer, 3. Posteriot banded layer and 4. Fibrillar layer. Asterix showing excrescence and arrow- attenuated endothelium X(×4200) nodule (asterisk) arising from the posterior banded layer is covered by attenuated endothelium (arrow)



In 2 cases of PBK, the DM was composed of 4 layers similar to FECD, Fig. 6.

Fig 6. 4 layers in PBK: thin PNBL and thick fibrillar (F) layer. No excrescence seen X 6000

In the remaining 3 stripping specimens, DM was less in thickness, approximately 8-9µm as compared to DM in FECD specimens. Moreover, no posterior nodules were present, Fig 7.

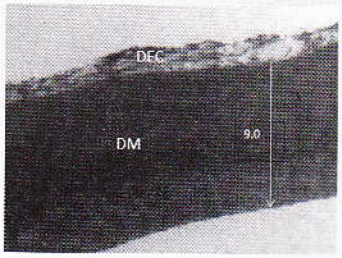
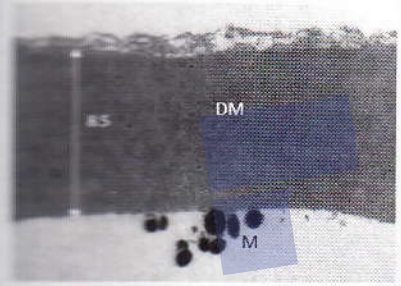


Fig 7. DM 9.0µm thick and Endothelial cells are completely degenerated and almost unremarkable X 4300



The endothelial cells were unremarkable or exhibited variable degree of degeneration, atrophy or loss. Large melanin granules were present on DM, Fig 8.

Fig 8. Transmission Electron microscopy showing degenerated endothelial cells (DEC) and a homogeneous DM 8.5µm thick. Melanin granules (M) are present on stromal side X 4300

Disintegrating endothelial cells left a large intercellular dropped out space containing debris and granular material, Fig 9.

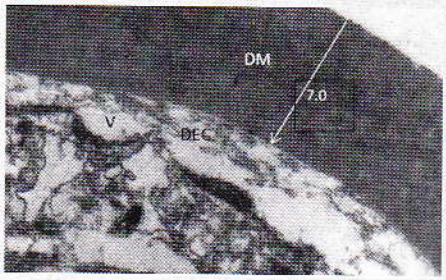


Fig 9. Degenerated endothelial cell (DEC) in PBK with a thinner DM, 7.0µm. Intracellular lacunar spaces (S), pigmented granules (P) X 6000

In addition, most of the intercellular junctions between the endothelial cells were loosened or absent due to the degeneration and loss of adjacent cells. Yet we observed few normal intercellular junction complexes in 2 samples of FECD, Fig10.

Fig 10. Normal intercellular junction (J) with dropped out spaces (S) in one cell X 6000

Higher magnification of TEM revealed the presence of increased number of lysosomes and vacuoles in the endothelium in both FECD and PBK.

This explains the over-production of enzymes which are responsible for the cytolysis on the endothelial cell membrane and eventually leading to the destruction of the architecture of the endothelium.

DISCUSSION

The primary defect of FECD is in the endothelium because HCEC loss, thickened DM and the presence of posterior nodules in the central cornea. In our study, we found that the pattern of DM changes consists of 4 layers in 2 cases of FECD and 2 cases of PBK. These layers were: Anterior banded fetal layer, Posterior non-banded layer (PNBL), Posterior banded layer (PBL) and Fibrillar layer.

The anterior banded layer was relatively constant and represented the fetal portion of DM. It was present in all cases. The banding of this layer is due to the presence of wide-spaced collagen. The PNBL is usually present in an otherwise normal cornea and thickens with age. It has been suggested that this may reflect abnormal endothelial function early in life with the production of the abnormal PBL rather than the normal PNBL. In FECD the corneal endothelium produces excessive amounts of basement membrane material of an abnormal composition resulting in the formation of a posterior collagenous layer. Extreme accumulations of this material created mushroom-like formations, guttatae, projecting into the anterior chamber. The initial manifestation in FECD is central guttatae.

In their study, Takeo Iwamoto and A. Gerard DeVoe(1971)[14] found that Descemet's membrane was markedly thickened in six cases and showed various structuralalterations. Five different regions could regularly be distinguished in the pathologic Descemet's membrane in all cases. From anterior to posterior they were (1) "anterior banded region" with 1,000 A-banded pattern, (2) "nonbanded region," without clear banding (these regions are seen in normal corneas); (3) "posterior banded region" filled with 1,000 A° -banded material ("warts" are formed by its partial backward protrusions); (4) "border region" composed of groups of "thin fibrils," "long-spacing bundles" of 1,000 A° periodicity with two type of banded pattern, and "basement membrane-like material"; and (5) "fibrillar region" which consists of "basement membrane-like material" and collagen fibrils. One case had warts located very posteriorly, and another, withno clear warts, but both could be interpreted as variations of the above structure.

The endothelium was thin, consisting mainly of two types of abnormal cells. Loosening of the functional complexes was common, and partial discontinuity of the endothelial cover was also seen. The Type 1 cell had cytoplasmic filaments, increased rough-surfaced endoplasmic reticulum (RER) and cytoplasmic processes, simulating fibroblasts. The Type 2 cell had elongated RER and lysosomes within a less dense cytoplasm, and was probably a degenerate form of the Type 1 cell.Based on their findings, the following hypothesis was proposed: For unknown reasons, possibly hereditary, the endothelial cell morphology and function become similar to those of fibroblasts and they start producing collagen fibrils and basement membrane-like material, forming the fibrillar region of Descemet's membrane. At the border region, the collagen fibrils disintegrate into thin fibrils and partly further transform into long-spacing bundles. These, together with basement membrane-like material, are finally incorporated into the posterior banded region. Acceleration of this process forms warts. Similar changes can be seen at the extreme periphery of the normal adult cornea as a physiological phenomenon. In our study, DM in FECD comprised of 4 layers,

except the "border layer" which was not seen in our study. In Iwamoto's study and our study both, loosening of intercellular junction complexes and degenerative changes in the endothelial cells were noted.

In 1982, William M. Bourne et al.[15] studied the ultrastructure of DM by TEM in corneal buttons removed from 11 phakic eyes with FECD. Abnormalities in DM consistent with abnormal endothelial function early in life (prior the age of 20) were present in all corneas. An abnormal fibrillar layer was thicker in those corneas with greater stromal and epithelial edema, possibly indicating that this first layer is formed mainly during period of endothelial decompensation. In our study, fibrillar layer is present in 2 FECD and 2 PBK cases, but we cannot comment whether it is the first layer laid out due to endothelial decompensation.

KevineZaniolo et al. (2012), in Montreal, Canada, evaluated HCEC isolated form corneas with FECD. The purpose of the study was to assess the feasibility of initiating primary cultures of corneal endothelial cells from patients suffering from FECD. They also evaluated which conditions yielded the best results for culture.Ultrastructure of DM revealed an abnormal Posterior Banded Layer (PBL) and a fibrillar layer. There were guttatae as well in the posterior banded layer. Details of a DM with large striated bodies of 0.11µm periodicity were present in the edge of the PBL and perpendicular to the surface. Similarly, in our study, the specimens revealed the above-defined layers, except we couldn't culture the endothelial cells for further proliferation.

The ultra-structural changes in FECD, for example, presence of lipid keratopathy, melanin granules in the stroma, increase in the thickness of DM, fibrils in the cytoplasm of the degenerating endothelial cells explain that the process is more chronic than in PBK.

CONCLUSION

With advancing diseases, the endothelial cells in FECD and PBK become increasingly dedifferentiated from their normal morphology as well as function. This alteration or metaplasia makes them appear more fibroblast-like cytologically with increased rough endoplasmic reticulum, cytoplasmic filaments, lysosomes, membrane-bound vacuoles, phagocytosed pigment granules and some desmosomal intercellular junctions. Increasingly degenerate changes occur with swollen mitochondria, widened intercellular spaces, larger vacuoles and pinocytic nuclei and death of many cells.From our study, we deduce that in FECD and PBK, there are ultra-structural changes: DM comprises of 4 layers: anterior banded fetal layer, posterior non-banded layer, posterior collagen layer and fibrillar layer. But the presence of lipid keratopathy, melanin granules, thickened DM, fibrils in the cytoplasm in FECD specimens denotes a more chronic process in FECD as compared to PBK, which appears to be short-termed activity.

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