Yonago Acta medica 2004;47:29–35

A Scanning Electron Microscopic Study of the Basal Surface of the Corneal Endothelium and the Stromal and Endothelial Surfaces of Descemet's Membrane in Rats

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The basal surface of the corneal endothelium and the stromal and endothelial surfaces of Descemet's membrane in rats were studied by scanning electron microscopy. We compared the fine structures of the two surfaces of Descemet's membrane both after sputter-coating with platinum and without sputter-coating. Fine structures were made clearly visible without metal coating by heating specimens to 300˚C during observation. After sputter-coating, both surfaces of Descemet's membrane were composed of granular substances, but their sizes were larger on the stromal side than on the endothelial side. Both surfaces of Descemet's membrane observed without sputter-coating were composed of fine fibrous structures showing a felt-like appearance, but their diameters were thicker on the stromal surface. These results may reflect a difference in collagen types between the two surfaces of Descemet's membrane.

Key words: corneal endothelium; Descemet's membrane; non-coating observation method; sputter-coating with platinum

Scanning electron microscopy (SEM) is a useful method for studying three-dimensional structures of tissues and cells. Since Blümcke and Morgenroth (1967) observed the three-dimensional structure of the corneal endothelium of normal adult rabbits, many researchers have studied the three-dimensional structure in normal or pathological corneas by SEM (Svedbregh and Bill, 1972; Sugita, 1976; Doughman et al., 1976; Melamed et al., 1980; MacCallum et al., 1983; Yamasaki and Inoué, 2001). However, these observations were restricted to the free surface of the endothelium. Descemet's membrane is located between the endothelium and stroma in the cornea. Since the membrane is firmly connected to the endothelium, it is difficult to observe the basal surface of the endothelium and the endothelial surface of the membrane by SEM. Thus, up until now, the three-dimensional architecture of the cornea could only be speculated from thin section images by transmission electron microscopy (TEM) (Hogan et al, 1971; Komai et al., 1990).

In this study, we tried to demonstrate the corneal surfaces in rats by the mechanical separation/SEM method instead of the previous cell-maceration/SEM method (Komai et al., 1990). In addition, we observed three-dimensional ultrastructures of both the endothelial and stromal sides of Descemet's membrane by the exfoliating method

Abbreviations: SEM, scanning electron microscopy; TEM, transmission electron microscopy

using surface tension (Inoué et al., 1984). Furthermore, we compared the ultrastructures of Descemet's membrane with or without metal coating (Osatake and Inoué, 1998). We report here some new findings in the ultrastructures of the basal surface of the endothelium as well as the stromal and endothelial surfaces of Descemet's membrane.

Materials and Methods

Animals

Adult Wistar rats of both sexes, weighing 170 to 270 g, were used in this study. All experiments conformed to the Association for Research in Vision and Ophthalmology Resolution on the Human Use of Animals in Research, and the Guidelines for Animal Experiments in the Tottori University Faculty of Medicine. Under sodium pentobarbital (40 to 50 mg/kg body weight) anesthesia administered intraperitoneally, the animals were perfused with a mixture of 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2, 160 mOsm/kg) through the left ventricle. The eyeballs were then enucleated, and each cornea was cut out at the limbus. The corneas were further immersed in the same fixative for additional 3 to 5 days.

Transmission electron microscopy (TEM)

The corneas were cut into small pieces and rinsed overnight in a buffer containing 0.2 M sucrose (pH 7.2, 430 mOsm/kg) followed by postfixation with 1% osmium tetroxide for 1 h. After block-staining in 1% uranyl acetate, they were dehydrated in a graded ethanol series and embedded in Epon through propylene oxide. Thin sections were obtained using a diamond knife and an ultramicrotome (Utracut UCT, Leica, Wien, Austria), and were examined with a transmission electron microscope (100 CX II, JEDL Ltd., Tokyo, Japan) operated at 80 kV after staining for connective tissues (Kajikawa et al., 1975).

SEM preparation for observing the basal surface of the corneal endothelium

After washing in 0.1 M phosphate buffer (pH 7.2, 210 mOsm/kg), fixed corneas were separated mechanically using a 26-gauge needle; the endothelial surface in each material was picked and scratched prudently by the fine needle. When the endothelial surface was finally detached from Descemet's membrane and turned over, we could

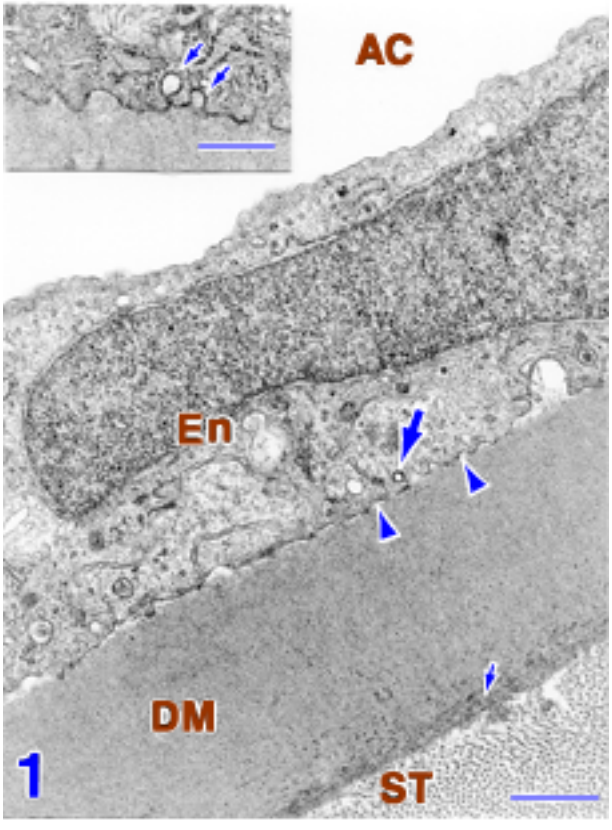

Fig. 1. TEM image of the cross-section of the rat corneal endothelium (En), Descemet's membrane (DM) and stroma (ST). The endothelial surface of Descemet's membrane has an irregular profile (arrowheads), reflecting the basal contour of the endothelium. Microfibrils (small arrow) are seen on the stromal side of Descemet's membrane, while they are not visible on the endothelial side. Pinocytotic vesicles (large arrow) are visible beneath the basal cell membrane of the endothelium. Bar = 1 μm. AC, anterior chamber. Inset: Higher magnification of the basal surface of the endothelium. Note pinocytic vesicles (arrows). Bar = 0.5 μm.

observe the basal surface of the endothelium. These specimens were then thoroughly washed in the buffer, postfixed in 1% osmium tetroxide for 1 h and conductive-stained with 2% tannic acid and 1% osmium tetroxide (Murakami, 1974). After dehydration through a graded ethanol series, they were finally freeze-dried by *t*-butyl alcohol (Inoué and Osatake, 1988). The dried specimens were observed with a scanning electron microscope (HFS-2ST, Hitachi Ltd., Tokyo) operated at 25 kV after sputter-coating with platinum.

SEM preparation for observing Descemet's membrane

The two surfaces of Descemet's membrane were exposed by the exfoliating method using surface tension (Inoué et al., 1984). The corneas were fixed by immersion with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2, 210 mOsm/kg) for 2 h at room temperature. The specimens were further treated with 0.1% osmium tetroxide in 0.1 M phosphate buffer for 12 h at room temperature. After a brief rinse in distilled water, they were dehydrated in a graded ethanol series up to a concentration of 100%. The dehydrated specimens were then thrown into distilled water. The specimens moved about on the surface and eventually sank. At this point, some parts of the endothelium and Descemet's membrane were separated by the surface tension produced between the water and the ethanol. The specimens were osmicated again with 1% osmium tetroxide in the same buffer for 20 min, rinsed with the buffer, and conductive-stained with a 2% tannic acid and 1% osmium tetroxide. After dehydration through a graded ethanol series again, they were finally freeze-dried by *t*-butyl alcohol. The dried specimens were observed with a Hitachi scanning electron microscope (HFS-2ST) operated at 25 kV after sputter-coating with platinum. Some specimens were observed without sputter-coating using a heating stage to 300˚C of a high resolution scanning microscope (UHS-T1, Hitachi Ltd.) operated at 25 kV (Osatake and Inoué, 1998).

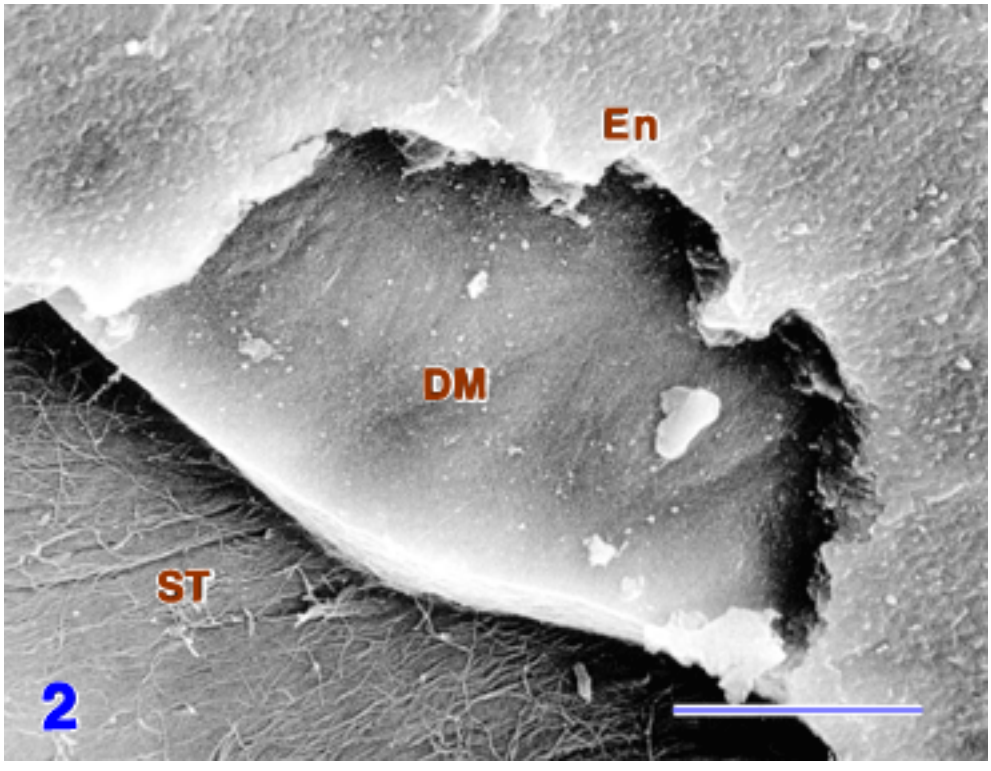

Fig. 2. SEM image of the mechanically-fractured cornea showing surfaces of the endothelium (En), Descemet's membrane (DM) and stroma (ST). Bar = 10 μm.

Results

TEM of the cornea

A Descemet's membrane sample of approximately 2.5 to 3 μm thick was observed between the endothelium and stroma (Fig. 1). The endothelial surface of the membrane showed an irregular profile, which corresponded to the undulation of the facing basal surface of the endothelium. The protruded part of the endothelium showed high electron-density. Pinocytotic vesicles were also visible beneath the basal cell membrane (Fig. 1, large arrow and inset). At the endothelial side, the membrane was composed of amorphous materials, whereas a high electron-density zone was noted at the stromal side. Aggregations of microfibrils were visible on the stromal side of the membrane (Fig. 1, small arrow), which appeared to be interwoven into the stroma and unite Descemet's membrane to the stroma, resulting in an unclear border between them.

SEM of the mechanically-fractured cornea

Surfaces of the endothelium, Descemet's membrane and stroma were directly observed on a mechanically-fractured cornea by SEM (Fig. 2). Using the mechanical separation method, lots of small wrinkles of approximately 100 to 150 nm in diameter and small openings of approximately 200 nm in diameter were seen on the basal surface of the endothelium (Fig. 3a). Uneven structures and small projections were seen on the endothelial surface of the membrane (Fig. 3b), which may correspond to the small wrinkles and openings.

SEM of Descemet's membrane

Descemet's membrane was successfully separated from the stroma by the exfoliating method using surface tension (Fig. 4). The endothelial surface of the membrane with platinum coating in a surface tension specimen was composed of relatively uniform, granular substances of approximately 10 to 58 nm in diam

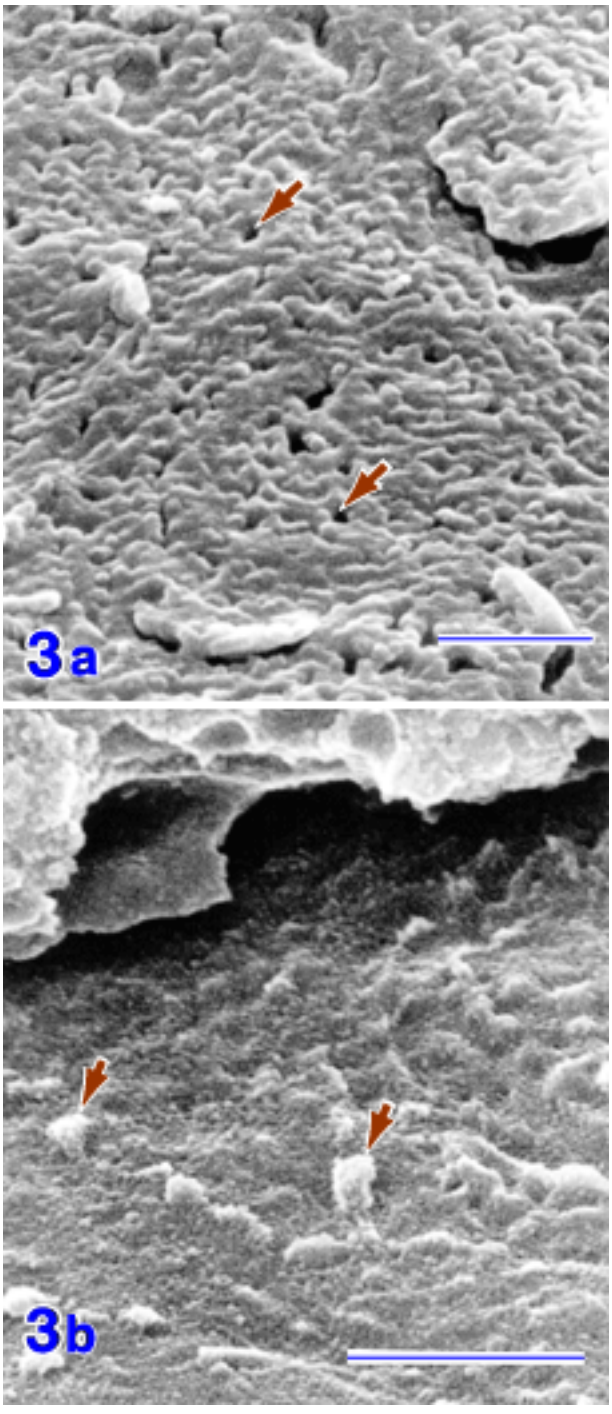

Fig. 3. SEM images of the basal surface of the endothelium (**a**) and the endothelial surface of Descemet's membrane (**b**) from the specimens prepared by the mechanical separation method.

a: The basal surface of the endothelium showing lots of small wrinkles and small openings (arrows). Bar = 2 μm.

b: The endothelial surface of Descemet's membrane showing uneven structures and small projectings (arrows), which may correspond to the small wrinkles and openings on the basal surface of the endothelium. Bar = 1 μm.

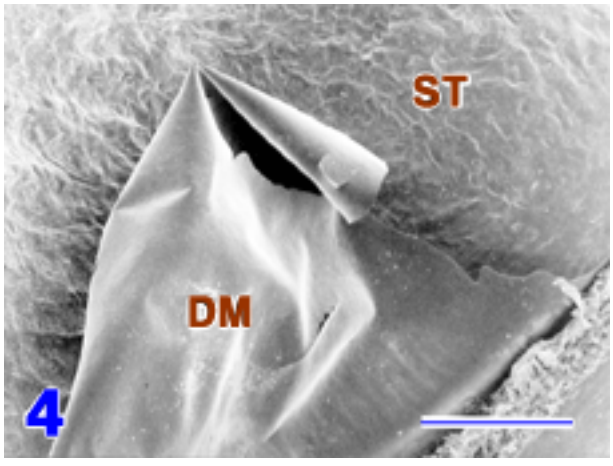

Fig. 4. Low-magnified SEM image of Descemet's membrane (DM) exfoliated from the stroma (ST) by the surface tension. Bar = 1 μm.

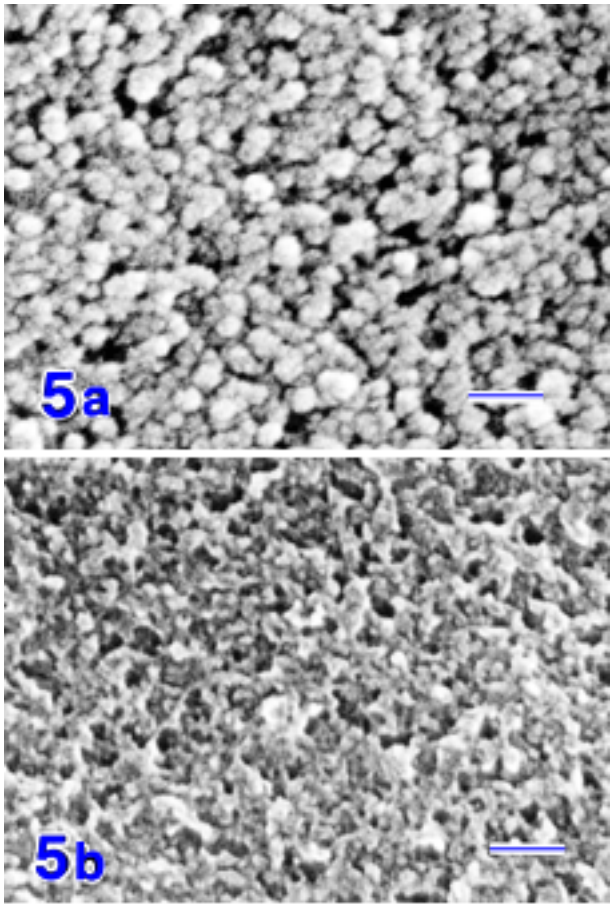

Fig. 5. SEM images of the endothelial surface of Descemet's membrane with platinum coating in a surface tension specimen (**a**) and without coating in a heating specimen (**b**). The metal-coated Descemet's membrane is composed of fine granular substances, whereas the uncoated Descemet's membrane shows a felt-like appearance with fine fibrous structures. Bar = 100 nm.

eter (30.0 ± 8.0 nm: mean ± SD, calculated on all prepared specimens) (Fig. 5a), whereas in the uncoated specimen, it showed a felt-like appearance with fibrous structures of approximately 15 to 20 nm in diameter (Fig. 5b).

On the other hand, the stromal surface of the membrane with platinum coating in a surface tension specimen was composed of relatively ill-balanced, larger-sized granular substances of approximately 26 to 78 nm in diameter (50.0 ± 12.8 nm: mean ± SD, calculated on all prepared specimens) (Fig. 6a), whereas in the uncoated specimen, it showed a felt-like appearance with fibrous structures of approximately 30 to 50 nm in diameter, which were thicker than that on the endothelial side (Fig. 6b).

Discussion

The undulation of the basal surface of the endothelium observed by TEM (Fig. 1) is supposed to correspond to small wrinkles on the basal surface of the endothelium observed by SEM (Fig. 3a). Small openings on the basal surface of the endothelium observed by SEM (Fig. 3a, arrows) are thought to correspond to orifices of pinocytotic vesicles beneath the basal cell membrane of the endothelium observed by TEM (Fig. 1). Pinocytotic vesicles are supposed to be concerned with the active pumping action of the endothelium, by which intracorneal fluid is pumped out into the anterior chamber when the fluid volume is increased.

In TEM images, the border between the stroma and Descemet's membrane is less distinct than that between the endothelium and the membrane, because of the presence of collagen fibrils within the stromal side of Descemet's membrane (Hogan et al., 1971; Komai et al., 1990). The present TEM study demonstrated the binding between the stroma and the membrane by the collagen fibrils (Fig. 1).

According to the three-dimensional observation of the basal lamina in pancreatic acinar cells, cardiac muscles and blood capillaries, each basal lamina consisted of globular materials of various sizes which were attached to or buried in a flat mesh-

work composed of fine filaments and amorphous substances (Sawada, 1981). In this study, Descemet's membrane with platinum coating in the surface tension specimen consisted of fine granular substances as observed by Sawada (1981) (Figs. 5a and 6a). However, the size of the granular substances obviously differed between the endothelial and stromal sides. These results suggest that their native sizes also differ between the two surfaces.

Descemet's membrane was mainly composed of type VIII collagen, partly by type IV and barely by type VI collagens (van der Rest and Garrone, 1991). Marshall et al. (1993) demonstrated, in an immunoelectron study, that type VIII collagen was abundant in the stromal side of Descemet's membrane and type IV collagen was rich in the endothelial side. They also showed that collagen types V and VI were present in the corneal stroma and its transitional zone to Descemet's membrane, for adhering the stroma to the membrane.

Our observation of Descemet's membrane without metal coating clarified that its endothelial surface was composed of felt-like fine filamentous substances (Fig. 5b), whereas the stromal surface was composed of thicker fibrous structures (Fig. 6b). These results may reflect a difference of the collagen types between the two surfaces of the membrane.

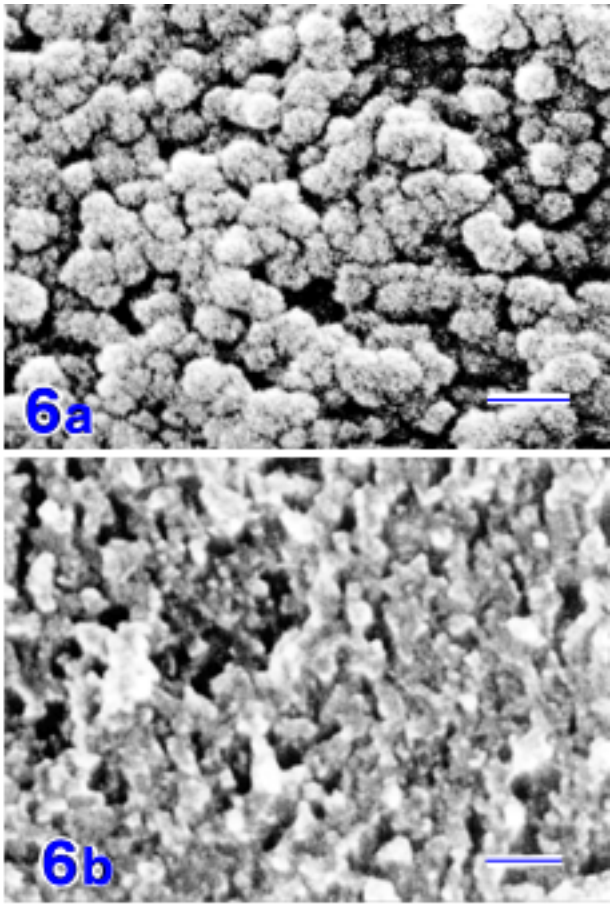

Fig. 6. SEM images of the stromal surface of Descemet's membrane with platinum coating in a surface tension specimen (**a**) and without coating in a heating specimen (**b**). The metal-coated Descemet's membrane is composed of fine granular substances of larger size, whereas the uncoated Descemet's membrane shows a felt-like appearance with thicker fibrous structures than on the endothelial side. Bar = 100 nm.

Acknowledgments: We express our sincere thanks to Professor Yoshitsugu Inoue, Division of Ophthalmology and Visual Science, Department of Medicine of Sensory and Motor Organs, School of Medicine, Tottori University Faculty of Medicine, for his generous help. We are also indebted to Mr. Hitoshi Osatake (Division of Morphological Analysis, Department of Functional, Morphological and Regulatory Science, School of Medicine, Tottori University Faculty of Medicine) for his technical help.

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Received March 3, 2004; accepted March 31, 2004

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