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Ultrastructure of the Posterior Corneal Stroma

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Purpose: To reinvestigate the ultrastructure of the posterior stroma of the human cornea and to correlate the findings with the stromal behavior after big-bubble creation.

Design: Observational consecutive 3-center case series.

Specimens: Fresh corneoscleral buttons from human donors (n = 19) and organ-cultured corneoscleral buttons (n = 10) obtained after Descemet's membrane endothelial keratoplasty.

Methods: Corneal specimens were divided into central (3 mm), mid peripheral (8 mm), and peripheral parts by trephination and processed for transmission electron microscopic and immunohistochemical analyses. A big bubble was created by air injection into the stroma of organ-cultured corneas before fixation.

Main Outcome Measures: The distance of keratocytes to Descemet's membrane, number of collagen lamellae between keratocytes and Descemet's membrane, diameter and arrangement of collagen fibrils, thickness of stromal lamella created by air injection, and immunopositivity for collagen types III, IV, and VI.

Results: Stromal keratocytes were observed at variable distances from Descemet's membrane, increasing from 1.5 to 12 μm (mean, 4.97±2.19 μm) in the central, 3.5 to 14 μm (mean, 8.03±2.47 μm) in the midperipheral, and 4.5 to 18 μm (mean, 9.77±2.90 μm) in the peripheral regions. The differences in mean distances were significant ($P < 0.0001$). The number of collagen lamellae between Descemet's membrane and most posterior keratocytes varied from 2 to 10 and the diameter of collagen fibrils averaged 23.5±1.8 nm and corresponded with that of the remaining stroma. A thin layer (0.5−1.0 μm thick) of randomly arranged, unaligned collagen fibers, which was positive for collagen types III and VI, was observed at the Descemet−stroma interface. The residual stromal sheet separated by air injection in 8 of 10 donor corneas varied in thickness from 4.5 to 27.5 μm, even within individual corneas (≤3-fold), and was composed of 5 to 11 collagen lamellae that revealed keratocytes on their anterior surface and in between.

Conclusions: Barring an anchoring zone of interwoven collagen fibers at the Descemet−stroma interface, the findings did not provide any evidence for the existence of a distinctive acellular pre-Descemet's stromal layer in the human cornea. The intrastromal cleavage plane after pneumodissection seems to be nonreproducibly determined by the intraindividually and interindividually variable distances of keratocytes to Descemet's membrane. *Ophthalmology 2014;■:1−7 © 2014 by the American Academy of Ophthalmology.*

With a more widespread application of new lamellar corneal transplantation techniques, such as deep anterior lamellar keratoplasty (DALK), Descemet's stripping automated endothelial keratoplasty, and Descemet membrane endothelial keratoplasty (DMEK), the structural and biomechanical characteristics of the posterior corneal stroma and Descemet's membrane (DM), as well as the DM−stroma interface become increasingly important. Detailed ultrastructural features of the DM−stroma interface have been previously described, including a thin intermediary meshwork of randomly arranged collagen fibrils projecting into the anterior DM zone and extracellular matrix complexes formed by keratoepithelin (transforming growth factor-β−induced) and collagen type VI, suggesting a firm connection between DM and the posterior stroma.[1−4] We have continued to learn about structural and biochemical characteristics of DM, which contains increased amounts of adhesive glycoproteins in its most anterior zone, the interfacial matrix, mediating stromal adherence, and about its physiologic cleavage plane after stripping, as well as its interindividual morphologic and biochemical variations preventing stripping in about 2% of donor corneas.[5−7]

Recently, the existence of a novel, well-defined, acellular layer of the pre-DM corneal stroma, which can be separated by air injection into the stroma (big-bubble technique),[8] has been reported.[9] This distinct layer was reported to measure 6 to 16 μm (mean, 10.15±3.6 μm) in width and was characterized to lack any keratocytes and to consist of 5 to 8 tightly packed collagen lamellae containing collagen fibers with a slightly thinner diameter (21.7±2.4 nm) than that of collagen fibers in the remaining stroma (24.2±2.6 nm). Immunohistochemistry showed a more pronounced staining of this layer for collagen types III, IV, and VI.[10] Although detailed analyses of the architecture of the corneal stroma

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have been published previously,[11–15] a distinctive pre-DM stromal layer had not been reported. Therefore, the description of this hypothesized new anatomic layer was critically commented on in the literature.[16–18] However, because structural features of the posterior cornea are likely relevant for lamellar surgical procedures, we performed a detailed ultrastructural reinvestigation of the posterior stroma of human corneas by combining corneoscleral tissue specimens and morphologic expertise from 3 different ophthalmologic laboratories and tried to correlate the ultrastructural findings collated in fresh intact corneas with observations made in organ-cultured corneas after big-bubble creation.

Materials and Methods

Tissue Specimens

For electron microscopic analysis, fresh corneoscleral tissue specimens (n = 5) were obtained from normal human donor eyes (mean age, 66.3±12.8 years) without any known ocular diseases, that were not suitable for transplantation, and were fixed within 10 hours after death. Another 5 fresh corneoscleral tissue specimens from human eyes (mean age, 59.8±14.9 years) enucleated for intraocular melanoma were obtained and fixed in transmission electron microscopy fixative[19] immediately after enucleation. In addition, 4 donor buttons (mean age, 52.0±10.5 years) within 10 hours of death stored in Optisol-GS at 4°C were obtained from eye banks (SightLife, Seattle, WA). Two buttons were stored for 6 days and another 2 for 15 days before fixation. Each participating ophthalmologic laboratory contributed ≥4 corneas each for ultrastructural analysis. All corneal specimens were divided into central (3 mm diameter), midperipheral (8 mm diameter), and peripheral parts by trephination after fixation to avoid any mechanical tissue damage and were further processed for transmission electron microscopy.

Another 10 corneal donor tissues (mean age, 68.5±8.9 years), which had been organ-cultured in Dulbecco's Modified Eagle's Medium containing streptomycin, penicillin, and fetal calf serum (Biochrom, Berlin, Germany) for about 4 weeks, were obtained after DMEK. In these corneal buttons, a big bubble was created by air injection into the stroma[8] using a 30-gauge needle before fixation and processing for light and transmission electron microscopy.

For immunohistochemistry, additional corneal tissues were obtained from 5 normal donor eyes (mean age, 67.6±10.3 years) without any known ocular disease within 10 hours after death.

Informed consent for tissue donation and use in research was obtained from the relatives and the study adhered to the tenets of the Declaration of Helsinki for experiments involving human tissue. Institutional review board/ethics committees at the institution of each laboratory provided approval for this study.

Transmission Electron Microscopy

Corneal specimens were fixed in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer, postfixed in 2% buffered osmium tetroxide, dehydrated in graded alcohol concentrations, and embedded in epoxy resin according to standard protocols. We stained 1-μm semithin sections for orientation with toluidine blue. Ultrathin sections (80 nm) were stained with uranyl acetate and lead citrate and examined with transmission electron microscopes (EM 906E or EM910, Carl Zeiss, Oberkochen, Germany; Tecnai G2 Spirit BT, FEI Company, Hillsboro, OR) in the 3 participating centers.

Images were captured and analyzed using CCD cameras (Olympus, Tokyo, Japan; Gatan Inc, Pleasanton, CA). Measurement of structural parameters (distance of keratocytes from DM, number of collagen lamellae between keratocytes and DM, diameter of collagen fibrils) were performed with automated image-processing systems and integrated software packages (Analysis, Soft Imaging Systems, Münster, Germany; Soft Imaging Solutions, Olympus; Digital Micrograph, Gatan Inc). Ten measurements were performed per corneal region (central, mid peripheral, peripheral) in 10 fresh and 4 short-term cultured corneal specimens, yielding a total of 420 measurements for each parameter. The thickness of the stromal layer created by air injection was measured at 20 measuring points in each of the organ-cultured corneal specimens (n = 10), yielding a total of 200 measurements.

Immunohistochemistry

For indirect immunofluorescence, corneal specimens were embedded in optimal cutting temperature compound and frozen in isopentane-cooled liquid nitrogen. Cryostat-cut sections (6 μm) were fixed in cold acetone for 10 minutes, blocked with 10% normal goat serum, and incubated in primary monoclonal antibodies against collagen type III (clone 1E7-D7; Millipore, Schwalbach, Germany), collagen type IV (clone 2F11; SouthernBiotech, Birmingham, AL), and collagen type VI (clone 3C4; Millipore) diluted in phosphate-buffered saline overnight at 4°C. Antibody binding was detected by Alexa 488-conjugated secondary antibodies (Molecular Probes, Eugene, OR) and nuclear counterstaining was performed with propidium iodide (Sigma-Aldrich, St Louis, MO). In negative control experiments, the primary antibodies were replaced by phosphate-buffered saline or equimolar concentrations of an irrelevant isotypic primary antibody.

Statistical Evaluation

Statistical evaluation was performed using SPSS version 21 (IBM Corp, Armonk, NY). The normal distribution of tested values was assessed by the Kolmogorov–Smirnov method. Differences between groups were evaluated by the *t*-test for independent samples. The significance level was set at $P = 0.05$.

Results

The structure of the pre-DM stroma did not differ from that of the central corneal stroma by light microscopic examination and did not reveal any demarcation of a distinct layer. Stromal keratocytes were observed at variable distances from DM, with focally close approximation. Transmission electron microscopy confirmed variable distances of stromal keratocytes from DM, which ranged from 1.5 to 12 μm (mean, 4.97±2.19 μm) in central areas, and 3.5 to 14 μm (mean, 8.03±2.47 μm) in midperipheral areas to 4.5 to 18 μm (mean, 9.77±2.90 μm) in peripheral areas (Fig 1). Thus, keratocytes approached DM ≤1.5 μm in the center of the cornea, while keeping greater distances with larger standard deviations in the midperipheral and peripheral cornea. The number of collagen lamellae between DM and the last row of keratocytes varied from 2 to 10 (mean, 5.65±2.02) in central areas, and 4 to 9 (mean, 6.31±1.38) in midperipheral areas, to 5 to 9 (mean, 6.62±1.17) in peripheral areas of the cornea. The differences between mean distances of keratocytes, which increased from central to midperipheral and peripheral corneal regions, were statistically significant ($P < 0.0001$; Fig 2A), whereas there were no differences between mean numbers of collagen lamellae (Fig 2B). The individual collagen fibrils in the posterior stroma, which were interspersed with long-spacing collagen fibers, had a uniform diameter