Association of the Gutta-Induced Microenvironment With Corneal Endothelial Cell Behavior and Demise in Fuchs Endothelial Corneal Dystrophy

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**IMPORTANCE** The number and size of guttae increase over time in Fuchs endothelial corneal dystrophy (FECD); however, the association between these physical parameters and disease pathogenesis is unclear.

**OBJECTIVE** To determine the role of guttae in corneal endothelial cell function.

**DESIGN, SETTINGS, AND PARTICIPANTS** In an in vitro model, cells from a human corneal endothelial cell line, HCENC-21T, were seeded on decellularized normal (n = 30) and FECD (n = 70) endothelial basement (Descemet) membranes (DMs). Normal human corneas were sent to our laboratory from 3 sources. The study took place at the Schepens Eye Research Institute, Massachusetts Eye and Ear, Boston, and was performed from September 2015 to July 2017. Normal DMs were obtained from 3 different tissue banks and FECD-DMs were obtained from patients undergoing endothelial keratoplasty in 2 departments.

**MAIN OUTCOMES AND MEASURES** Endothelial cell shape, growth, and migration were assessed by live-cell imaging, and gene expression analysis as a function of guttae diameter was assessed by laser capture microscopy.

**RESULTS** Mean (SD) age of normal-DMs donors was 65.6 (4.4) years (16 women [53%]), and mean (SD) age of FECD-DMs donors was 68.9 (10.6) years (43 women [61%]). Cells covered a greater area (mean [SD], 97.7% [8.5%]) with a greater mean (SD) number of cells (2083 [153] cells/mm²) on the normal DMs compared with the FECD DMs (72.8% [11%], P = .02 and 1541 [221] cells/mm² 221/mm², P = .01, respectively). Differences in endothelial cell growth over guttae were observed on FECD DMs depending on the guttae diameter. Guttae with a mean (SD) diameter of 10.5 (2.9) μm did not impede cell growth, whereas those with a diameter of 21.1 (4.9) μm were covered only by the cell cytoplasm. Guttae with the largest mean (SD) diameter, 31.8 (3.8) μm, were not covered by cells, which instead surrounded them in a rosette pattern. Moreover, cells adjacent to large guttae upregulated αSMA, N-cadherin, Snail1, and NOX4 genes compared with ones grown on normal DMs or small guttae. Furthermore, large guttae induced TUNEL-positive apoptosis in a rosette pattern, similar to ex vivo FECD specimens.

**CONCLUSIONS AND RELEVANCE** These findings highlight the important role of guttae in endothelial cell growth, migration, and survival. These data suggest that cell therapy procedures in FECD might be guided by the diameter of the host guttae if subsequent clinical studies confirm these laboratory findings.

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The corneal endothelium, derived from the neural crest, has important roles in corneal transparency. Human corneal endothelial cells (HCEncs) are arrested in the G1 phase and thus have limited proliferative ability. Human corneal endothelial cells decrease at a rate of 0.6% annually without affecting corneal transparency. However, severe damage to the corneal endothelium leads to an irreversible reduction of endothelial function, corneal edema, and loss of vision. Fuchs endothelial corneal dystrophy (FECD), estimated to affect 4% of the US population, is the primary cause of endogenous corneal endothelial degeneration, often leading to corneal transplantation. Fuchs endothelial corneal dystrophy is characterized by decreased endothelial cell density and formation of extracellular matrix (ECM) excrescences, called guttae, on the posterior Descemet membrane (DM), causing light scattering and thereby glare and visual problems.

The DM is a specialized basement membrane secreted by the CEnCs throughout the lifetime of an individual. The DM acquires a complex lamellar structure during fetal development and thickens from 3 μm at birth to 8 to 10 μm in adulthood; it is composed of various collagen types, glycoproteins, and proteoglycans organized in 2 layers. In FECD, the DM homeostasis is compromised, as evidenced by the formation of guttae along with enhanced thickening and the disorganization of ECM; however, the effect of guttae on endothelial cell function is unclear. Specifically, it is not known whether aberrant production of ECM has an effect on the intracellular findings seen in FECD pathobiology.

Fuchs endothelial corneal dystrophy is treated by endothelial keratoplasty, which is limited by the risk of rejection and shortage of donor corneas for grafting. Therefore, new technologies, such as cell-based therapy, are being explored. Several animal models have been used to develop therapeutic approaches based on the proliferation of grafted CEnCs; however, to our knowledge, a reliable model of FECD has not been established. An in vitro topographical model has shown that the size and density of guttae, which increase in late-stage FECD, may negatively affect the restoration of the HCEnc monolayer after corneal cell injection. However, this model used synthetic guttae and did not account for the actual guttage composition. Thus, to clarify the role of guttae in the pathogenesis of FECD and potential cell-based therapies, we investigated the properties of externally seeded normal human endothelial cells on guttae from FECD-affected corneas.

Methods

Study Design

Thirty normal corneas isolated from cadaveric donors and DMs obtained from 70 patients with FECD undergoing DM endothelial keratoplasty surgery were used. Immortalized HCEncs (HCEnc-21T) were seeded on normal DMs and FECD-DMs and were cultivated for 7 days. The study design is described in the Figure.

The mean (SD) age of normal DMs donors was 65.6 (4.4) years, and the mean (SD) age of FECD-DM donors was 68.9 (10.6) years. Female donors represented 54% in normal-DM group and 61% in FECD-DM group.

Collection of Human Corneal Endothelium Specimens

This study was conducted in accordance with the Declaration of Helsinki and approved by the institutional review board of Massachusetts Eye and Ear Institute, Boston. Donors as well as the next of kin of deceased donors provided informed written consent for the eye donations for research. Normal human corneas were obtained from the Northeast Pennsylvania Lions Eye Bank (Bethlehem, Pennsylvania), SightLife (Seattle, Washington), and Eversight (Chicago, Illinois) and delivered to the laboratory in transport medium (Optisol-GS; Bausch and Lomb).

The DM was isolated from normal donors by dissection and punching with an 8.00-mm trephine. Normal cornea endothelial DMs (N-DMs) were decellularized in ethylenediaminetetraacetic acid, 0.2%, for 3 minutes at 37°C, followed by gentle trituration using a fire-polished Pasteur pipette. The FECD-DMs were obtained from patients with FECD who underwent routine DM endothelial keratoplasty at the Massachusetts Eye and Ear Infirmary (Boston, Massachusetts) and Price Vision Group (Indianapolis, Indiana). After FECD-DM removal, the tissues were immediately placed in Optisol-GS storage medium at 4°C. The CEnCs were removed from the FECD-DMs as described for N-DMs. The FECD-DMs were examined before seeding the cells; DMs with tears or with several pieces were excluded.

Corneal Endothelial Cell Culture

Immortalized human corneal endothelial HCEnc-21T cells were cultured for 7 days in a supplemented Chen medium (OptiMEM-I; Invitrogen). The HCEncs were subcultured by detaching cells with trypsin, 0.05% (Invitrogen), for 5 minutes at 37°C. The HCEnc-21T cell number and viability were determined using an automatic cell counter (Countess, Life Technologies) and trypsin blue dye exclusion, respectively. Then, 2000 cells were plated on N-DM or FECD-DM stromal side down in 12-well cell culture inserts (3.0-μm pore size, positron emission tomography track-etched membrane; BD Falcon; Figure, A).

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Imaging of Endothelial Cell–Gutta Interactions

Cell morphology, growth, migration patterns, migration speed, and gutttae diameter were analyzed by phase-contrast microscopy (Leica DM IL LED) and live cell imaging (N-DMs, n = 8; FECD-DMs, n = 11) using an epifluorescence microscope (Leica DMI 6000B) connected to a Leica DFC350FX camera (Leica Microsystems). Phase-contrast and bright-field cell images were obtained using ×10 and ×20 objectives (numerical aperture: 0.25) and analyzed using ImageJ (National Institutes of Health, Bethesda, Maryland; Figure, A). The diameter of individual gutttae was calculated from 5 radial measurements of the top of an individual gutta. Cells were monitored by live-cell imaging, every 30 seconds for 10 hours. Raw cell migration tracks were plotted for time-lapse images using the Particle Analysis Manual Tracking plugin for ImageJ.

Cell Number, Guttae Coverage, and Apoptosis Analyses
To examine the cell density and percentage of cell coverage, HCEnc-21T cells grown on FECD-DMs (n = 19) and N-DMs (n = 6) for 7 days were analyzed as described previously.18,19
Both cell density and percentage of cell coverage were determined using ImageJ by analyzing the mean number of cells in 5 images from the center and periphery of the DM (each image represented a surface area of 1 mm²). Growth properties were analyzed by merging actin and PI staining with bright field images.

Apoptosis was measured using the terminal deoxynucleotidyl transferase deoxyuridine 5'-triphosphat nick-end labeling (TUNEL) assay (in situ Cell Death Detection Kit, Roche Diagnostics) according to the manufacturer's instructions (N-DMs, n = 7; FECD-DMs n = 5). Digital images were obtained using a spectral photometric confocal microscope (Leica DM6000S with LCS1.3.1 software; Leica Camera AG). The number of apoptotic cells per millimeters squared was calculated, and the correlation between apoptosis and guttae diameter was evaluated (Figure, A).

Isolation of HCEnc RNA Grown on DMs Using Laser Microdissection
The HCEnc-21T cells were grown on N-DMs (n = 6) and FECD-DMs (n = 15) for 7 days. The HCEnc-21T cells on FECD-DMs that grew around the small (<15 μm), medium (15-30 μm), and large (>30 μm) guttae were isolated using laser capture microdissection, and pooled in separate vials based on guttæ diameter (Model AS LMD; Leica). The specimens with small, medium, or large guttæ from all DMs were pooled for RNA extraction. Cells grown on N-DMs were also isolated by laser capture microdissection using cuts of similar diameters as those for FECD-DM, and the pieces were pooled for RNA extraction. A Leica LMD 6000 microscope equipped with Leica laser capture microdissection software, version 6.7.1.3952, was used to select areas for laser capture microdissection (Figure, B).

Analysis of Gene Expression in HCEncs Grown on DMs Quantitative Polymerase Chain Reaction of HCEncs Grown on DMs Without Laser Capture
The HCEnc-21T cells were lysed with TRIzol (Invitrogen), and RNA was extracted using the RNeasy Micro Kit and in-column DNase I digestion (Qiagen) according to the manufacturer's protocol. RNA quality and quantity were assessed using a NanoDrop (LabTech International), and 2 μg of total RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real-time polymerase chain reaction (Table) was performed as previously described.20 The expression levels of ATPase Na⁺/K⁺ transporting subunit α1 (ATP1A1), carbonic anhydrase 2 (CA2), clusterin, fibronectin, and vimentin were compared between cells grown on N-DMs (n = 16) and FECD-DMs (n = 25).

Quantitative Polymerase Chain Reaction of HCEncs Grown on DMs With Laser Capture
A mean of 125 cuts were performed per DM, allowing for the isolation of each gutta surrounded by CEnCs. Levels of N-cadherin, snail1, α-SMA, p21, p16, and nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) were compared between the N-DM (n = 6) and the 3 pooled subgroups of FECD-DM (n = 15) (small, <15 μm; medium, 15-30 μm; and large, >30 μm guttae). Expression levels were normalized to that of GAPDH. Template-free reactions were performed for each marker as a negative control. Relative expression was calculated by subtracting the normalized Ct values of N-DM-grown cells from those of the FECD-DM-grown cells using the 2ΔΔCT method.

Statistical Analysis
The results are presented as means (SD) of at least 6 experiments performed in triplicate. Differences between the samples were evaluated using GraphPad Prism7 by a 2-tailed t test. A 2-sided P value of less than .05 was considered statistically significant.

Results
Gutta Diameter in FECD Specimens
Guttæ were clinically visible by slitlamp examination (eFigure 1A in the Supplement). By in vivo confocal microscopy (eFigure 1B in the Supplement), guttæ appeared as bright spots surrounded by dark circular rings with interspersed cells. The CEnCs showed a decrease in density with cellular pleomorphism and polymegathism. Moreover, guttæ showing a characteristic dome shape with flattened and slightly indented top were detected by TEM (eFigure 1C in the Supplement).

The FECD-DMs (eFigure 1D in the Supplement) were de-cellularized and analyzed by bright field microscopy. Depending on the disease stage or progression, FECD-DM showed guttæ with a wide range of diameters (mean [SD], 27.2 [15.4] μm; range, 4-80 μm). The guttæ were larger in the center of the DM (38.3 [12.0] μm, eFigure 1D in the Supplement) than in the periphery of the cornea at the 8.00-mm DM edge (14.6 [6.4] μm; P < .001; eFigure 1D in the Supplement). There was less space between guttæ in the center of the cornea (5.4 [3.8] μm, eFigure 1D in the Supplement) than in the periphery at the 8.00-mm DM edge (106.3 [63.6] μm; P < .001, eFigure 1D in the Supplement).

Table. Assay Identification and Probe Sequence

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Endothelial Cell Behavior Relative to Guttae on FECD-DM

Normal HCEnCs were seeded directly on the N-DM and FECD-DM and cultivated for several days in Chen medium (Figure). Direct visualization by time-lapse microscopy revealed that the cells seeded on the N-DM had a greater mean migration rate on day 0 than that of cells seeded on the FECD-DM (0.35 μm/min vs 0.15 μm/min; \( P = .001 \)). On day 4, the migration rate was lower for cells grown on N-DM than for cells grown on FECD-DM (0.09 μm/min vs 0.22 μm/min; \( P = .001 \)), suggesting a delay in cellular attachment to FECD-DM.

On day 7, cells seeded on both N-DM and FECD-DM did not show any movement, revealing that they formed compact monolayers. After 7 days of culture, using propidium iodide and actin staining, we observed 3 distinct patterns of cell growth related to the guttae on FECD-DM during monolayer formation. In the first pattern, small guttae (mean [SD], 10.5 [2.9] μm) did not impede cell growth, leading to full coverage of guttae by cells during monolayer formation. In the second pattern, medium-sized guttae (mean [SD], 21.1 [4.9] μm) were covered with cells exhibited an elongated cytoplasm, while the nuclei remained at the base of the guttae, creating a rosette-like formation (eFigure 2A, B in the Supplement). In the third pattern, for large guttae (mean [SD], 31.8 [3.8] μm), both cell cytoplasm and nuclei were clustered around the base, and the apices of the guttae were not covered by cells (eFigure 2A, B in the Supplement). Cells surrounded the guttae in a clear rosette pattern, as observed in FECD ex vivo specimens. Diameters differed among groups of guttae (mean [SD] of small guttae, 10.5 [2.9] μm; medium guttae 21.1 [4.9] μm; large guttae 31.8 [3.8] μm; \( P < .001 \); eFigure 2C in the Supplement).

Cell Growth on N-DM and FECD-DM

The HCEnC growth properties on N-DM and FECD-DM after 7 days of culture were compared. Cell density was greater for N-DM (mean [SD], 2083 [153] cell/mm²) than for FECD-DM (mean [SD], 26.3 [14.2] cells/mm²) was greater than that on the N-DM (mean [SD], 0.4 [0.5] cells/mm²; \( P < .001 \)). The number of apoptotic cells surrounding the large guttae was increased to 48.9 [13.2] cells/mm² (eFigure 4C in the Supplement), similar to observations in ex vivo FECD samples (eFigure 4D in the Supplement), with a mean (SD) of 49% (9%) of the endothelial cells closely associated with the guttae being apoptotic, compared with only 20% (5%) of the endothelial cells not immediately adjacent to any guttae being apoptotic (\( P = .02 \), eFigure 4D in the Supplement).

Discussion

In this study, we showed that guttae were associated with inducing the phenotypic footprint of the degenerating CEnCs seen in FECD, clarifying the origins of the aberrant cell–ECM interactions in FECD. Capturing normal cell and abnormal ECM interactions using live-cell imaging, and further delineating the expression of FECD-related genes in cells associated with individual guttae using laser capture, we determined that guttae induce a stress response, senescence, the EMT phenotype, and cellular apoptosis in a size-dependent manner. A time-course analysis of HCEnC-2IT growth on FECD-DM revealed that small guttae did not inhibit monolayer formation, and there were no changes in the cellular phenotype (pattern 1). However, medium-sized guttae led to the formation of a cellular rosette-like structure, with stretching of the cellular cytoplasm over the tops of the guttae and an upregulation of both EnMT and senescence markers (pattern 2). Finally, the large guttae led to the rosette formation as seen in pattern 3 and induced EnMT, senescence, and apoptosis, indicating that these unique dome-shaped excrescences, by increasing in size, create an escalating perturbation of cell physiology.

This study highlights the critical role of guttae in endothelial degeneration. In fact, the primary pathobiology of FECD may lay in the formation of guttae because they have a capacity to sicken completely healthy cells. However, the fact that guttae did not induce all intercellular perturbations,
such as deficient antioxidant capacity, DNA damage, and mitochondrial dysfunction. It is likely that the primary abnormality in the cells renders them susceptible to various endogenous or exogenous stresses, causing the secretion of an aberrant ECM in the form of guttae, which after achieving the threshold diameter, create a toxic environment contributing to endothelial decompensation. Therefore, the aberrant ECM creates an additional factor that contributes to the vicious cycle in FECD pathogenesis, and might be secondary to a disturbed cellular synthetic ability among other preceding factors.

Importantly, our results show that the diameter of the guttae is an important determinant of cell behavior, consistent with the results of Rizwan et al indicating that the variable size and spacing of synthetic guttae was detrimental to cell monolayer formation. In this study, because we preserved the composition of the native guttae, we were also able to study the associations of an abnormal basement membrane microenvironment, in addition to size, with cell function. It is likely that specific factors present in large guttae influence FECD-related protein expression. Similarly, Xia et al suggested that structural and compositional changes in the FECD-DM affect cell behavior. Further proteomic analyses of individual guttae, based on size, might further elucidate the specific causes of the toxic environment in FECD.

Strengths and Limitations
A limitation of this study was the use of diameter as an indicator of volume. Rizwan et al analyzed the volume of the guttae. Future studies should obtain accurate estimates of volume. One advantage of our study is the use of real FECD specimens with randomly distributed guttae; previous synthetic models used regularly spaced guttae.

Our results may contribute to the design of corneal endothelial cell therapies. The development of in vitro techniques to stimulate the migration and proliferation of cultured HCEncCs has led to a paradigm shift where it is hoped that the endothelium could be rehabilitated without an allogenic donor. Cell therapies and factors that promote cell regeneration have been analyzed in rabbit, cat, and monkey models, where cellular injury is usually created by cell scraping, cryotherapy, or DM stripping. To our knowledge, guttae formation has not been detected in large animal models, limiting the ability to translate findings to patients with FECD.

In primary descemetorhexis without grafting, removing abnormal cellular matrices removes contact inhibition and induces the migration of healthy peripheral cells toward the center. Although regression of the corneal edema has been reported, visual outcomes are highly unpredictable, with irregular corneal astigmatism and posterior stromal opacities around the edges of the descemetorhexis. We did not observe an upregulation of EnMT, senescence markers, and apoptosis around small guttae, suggesting that not all areas of the FECD-DM have a harmful effect on cells. Therefore, endothelial cell injection should be guided by the guttae diameter, either by removing larger guttae or by treating patients before large guttae develop, without necessarily removing the DM.

Conclusions
Fuchs endothelial corneal dystrophy has been clinically graded using guttae number and area for decades. However, to our knowledge, guttae size has never been linked to the pathogenesis of FECD. Using LCM to isolate specific genes around guttae of varying diameters, our results highlight the central role of the guttae in the pathogenesis of FECD. Further studies are needed to determine the exact composition of the large guttae responsible for the stress, senescence, and apoptosis of the cells. In addition, if subsequent clinical studies confirm these laboratory findings, this study suggests that cell-based therapy should be guided by guttae diameter, and patients should be treated before large guttae appear, or guttae should be removed before procedures are performed.


