Human Corneal Anatomy Redefined
A Novel Pre-Descemet’s Layer (Dua’s Layer)

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Purpose: To define and characterize a novel pre-Descemet’s layer in the human cornea.

Design: Clinical and experimental study.

Participants: We included 31 human donor sclerocorneal discs, including 6 controls (mean age, 77.7 years).

Methods: Air was injected into the stroma of donor whole globes (n = 4) and sclerocorneal discs (n = 21) as in the clinical deep anterior lamellar keratoplasty procedure with the big bubble (BB) technique. The following experiments were performed: (1) creation of BB followed by peeling of the Descemet’s membrane (DM); (2) peeling off of the DM followed by creation of the BB, and (3) creation of the BB and continued inflation until the bubble popped to measure the popping pressure. Tissue obtained from these experiments was subjected to histologic examination.

Main Outcome Measures: Demonstration of a novel pre-Descemet’s layer (Dua’s layer) in the human cornea.

Results: Three types of BB were obtained. Type-1, is a well-circumscribed, central dome-shaped elevation up to 8.5 mm in diameter (n = 14). Type-2, is a thin-walled, large BB of maximum 10.5 mm diameter, which always started at the periphery, enlarging centrally to form a large BB (n = 5), and a mixed type (n = 3). With type-1 BB, unlike type-2 BB, it was possible to peel off DM completely without deflating the BB, indicating the presence of an additional layer of tissue. A type-1 BB could be created after first peeling off the DM (n = 5), confirming that DM was not essential to create a type-1 BB. The popping pressure was 1.45 bar and 0.6 bar for type-1 BB and type-2 BB, respectively. Histology confirmed that the cleavage occurred beyond the last row of keratocytes. This layer was acellular, measured 10.15±3.6 microns composed of 5 to 8 lamellae of predominantly type-1 collagen bundles arranged in transverse, longitudinal, and oblique directions.

Conclusions: There exists a novel, well-defined, acellular, strong layer in the pre-Descemet’s cornea. This separates along the last row of keratocytes in most cases performed with the BB technique. Its recognition will have considerable impact on posterior corneal surgery and the understanding of corneal biomechanics and posterior corneal pathology such as acute hydrops, Descemetocle and pre-Descemet’s dystrophies.

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The human cornea is made of the epithelium and Bowman’s zone anteriorly and the endothelium with its basement membrane, the Descemet’s membrane (DM), posteriorly, sandwiching the stroma. The anterior stroma is more compact than the posterior and differs in the composition of proteoglycans.1-3

Contemporary corneal transplantation involves selective replacement of the affected layer. Deep anterior lamellar keratoplasty (DALK) allows replacement of affected stroma while retaining the recipient’s healthy DM and endothelium,4,5 thus eliminating the risk of endothelial rejection. The DM can be separated from the stroma by injecting air, viscoelastic, or saline.4-9 Collectively, these techniques are referred to as “Descemet’s baring techniques,” where it is claimed that the cleavage occurs at a plane that enables the DM to be laid bare.8,9 The most popular technique is the big bubble (BB) technique5 wherein air is injected into the corneal stroma to affect a separation of the DM from the posterior stroma. Air accumulates between these 2 layers in the form of a BB, hence the name. Based on our clinical experience with DALK we had hypothesized the existence of a “pre-Descemet’s posterior stromal layer” and presented pilot data at the annual congresses of Societa Italiana Cellule Staminalie Superficie Oculare and the Royal College of Ophthalmologists, UK, in 2007.

In this study, we examined the plane of cleavage ex vivo in human donor whole eyes and sclerocorneal discs by injecting air into the corneal stroma, as is done during the clinical DALK procedure, and present conclusive data to demonstrate the presence of a well-defined, hitherto unknown layer in the human posterior cornea. We have termed this layer Dua’s layer (DL).
Methods

The following experiments were performed on human eye bank donor eyes:

1. creation of the BB followed by peeling off of the DM;
2. peeling off of the DM followed by creation of the BB; and
3. reation of the BB and continued inflation till the bubble popped to measure the popping pressure.

Tissue samples obtained from the above experiments were subjected to histologic examination by light, transmission and scanning electron microscopy (SEM) and immunohistochemistry.

Air Injection

Air was injected through the epithelial surface into the stroma of donor whole globes (n = 4) and sclerocorneal discs (n = 8) and from the endothelial surface of sclerocorneal discs (n = 13). A 30-gauge needle was inserted from the limbus into the mid-peripheral stroma. For injection from the epithelial surface, donor sclerocorneal discs were mounted on a Barron artificial anterior chamber (Katena, Denville, NJ). In total, we used 25 eyes (4 whole globes [2 donors] and 21 sclerocorneal discs [17 donors]). The causes of death were infections (n = 5), cardiac related (n = 4), cancer (n = 3), and others (n = 7). Before use, all sclerocorneal discs were maintained in organ culture in Eagle’s minimum essential medium with 2% fetal bovine serum for 4 to 8 weeks after death. Whole globes were obtained within 48 hours of death and, after injection of air, each sclerocorneal disc was excised and the cornea examined from the posterior surface for presence of a BB. All bubbles were measured with surgical calipers.

DM Peeling

With the BB facing up, the edge of the DM close to the sclerocorneal junction was scratched with a crescent blade or tip of a forceps. Thus lifted, the edge of the DM was grasped with a pair of forceps and peeled centrally up and across the BB. Occasionally, the DM tore and came off in small strips. It was possible to pick up the edge from another peripheral site to remove all of the DM.

Measuring Popping Pressure

A standard, calibrated pressure gauge (range, 0–2.5 bar) was connected to the side arm of a 3-way cannula. The needle for injection was attached to 1 end of the cannula and the syringe to the other. The piston of the syringe was steadily pushed to increase the pressure until the BB popped. The pressure exerted on the air in the syringe during injection was read directly off the gauge. After the bubble popped, the layer of tissue was grasped with a forceps and an attempt was made to mechanically peel this layer off the periphery of the cornea in 4 samples.

Sample Preparation

Cornea with BB. For immunostaining, the BB was deflated by aspirating the air with a needle inserted through the stroma into the bubble and the space refilled with optimal cutting temperature compound before snap-freezing in liquid nitrogen. For electron microscopy, the entire cornea with the bubble was immersed in 2.5% buffered glutaraldehyde.

Electron Microscopy

Tissue samples were prepared for SEM and transmission electron microscopy after standard procedures (Appendix 1, available online at http://aaojournal.org). We carried out SEM using a JSM 840 scanning electron microscope (JEOL, Herts, UK) and transmission electron microscopy with a JEOL 1010 microscope (JEOL).

Immunostaining

Frozen sections of tissues (Leica cryomicrotome; Wetzlar, Germany) obtained from the above experiments were stained with fluorescent dyes for collagens I, IV, V, and VI and the proteoglycans lumican, mimecan, and decorin. The adhesion molecule CD34 and 4′,6-diamidino-2-phenylindole (a fluorescent stain for nuclear DNA) were used to detect cell nuclei and keratocytes, respectively (Appendix 1).

Controls

Human donor cornea samples (n = 3) maintained in Eagle’s minimum essential medium without air injection were used for comparing the number of stromal layers in a given width of cornea compared with the DL.

In another 3 sclerocorneal discs, after obtaining a type-1 BB, the posterior wall of the type-1 BB was excised along its circumference and further injection of air carried out to determine whether another BB could be created.

Results

The average age of donors was 77.7 years (range, 53–94 years; median, 82 years). There were 10 females and 9 males. A BB was obtained in all 4 whole globes and in 18 of the 21 discs. Three types of BB were obtained. Type 1 is a well-circumscribed, central dome–shaped elevation measuring 7 to 8.5 mm in diameter (Fig 1A, B, video clip 1), which always started in the center of the cornea and enlarged circumferentially toward the periphery (n = 14). Type 2 is a thin-walled, large BB of maximum 10.5 mm diameter (Fig 1C, D, video clip 2), which, in contrast, always started as 1 or 2 small bubbles at the periphery, enlarging centrally (coalescing) to form a large BB (n = 5). The mixed type is a primary BB as in type 1, but with ≥1 smaller secondary bubbles as in type 2 (n = 3; Fig 1E, F).

With the type-1 BB it was possible to peel off the DM completely without deflating the BB every time (Fig 2A, B video clip 3), indicating that the posterior wall of the BB was made of the DM and an additional layer of tissue. With type-2 BB, peeling the DM resulted in deflation of the bubble as soon as the edge of the BB was reached, indicating that all the air was beneath DM with no additional layer (video clip 4). With the mixed type BB, when the edge of the peeled DM reached the edge of the secondary bubble(s), the secondary bubble(s) deflated and the DM was found to be continuous with the wall of the secondary bubble(s). With continued peeling, the DM could be removed from the surface of the primary BB without deflating it, as seen in type-1 BB.

In 5 sclerocorneal discs, a type-2 BB was first obtained, which collapsed when the DM was peeled off, but in the same sclerocorneal discs, upon further injection of air, a type-1 BB could be created, indicating that the presence of the DM was not essential to the formation of a type-1 BB (video clip 4). In the 4 instances where sustained air pressure was applied to push the type-1 BB to its maximum dimension, the bubble extended to a 9-mm diameter and popped with a sound. For 2 of these bubbles, the popping pressure was measured at 1.4 and 1.5 bar. For a type-2 BB, the popping pressure was 0.6 bar. The DL could not be mechanically peeled off beyond a 9-mm diameter, but tension on the DL induced fine wrinkles or striae that could be seen extending across the edge of the type-1 BB toward the periphery of the cornea (Fig 3A, B, video clip 5). Such striae could also be seen in type-1 BBs.
obtained after peeling off the DM, when the bubble was forcefully inflated (in the experiments designed to pop the bubble).

After obtaining a type-1 BB, when the posterior wall of the type-1 BB was excised along its circumference (n = 3 controls) and further injection of air carried out, in no case was another BB created, indicating that the type-1 BB is not a random separation of a few layers of the deep corneal stroma (video clip 5).

**Histopathology**

Light and electron microscopy revealed that the posterior wall of the type-1 and mixed type (primary) BBs was consistently made of DM and DL (Fig 4A, B); type-2 BB was made of DM only (Fig 4C), and those created after removing the DM were made of DL only (Fig 4D). When the DM was partially peeled off, the difference between DL

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**Figure 1.** All images show the sclerocorneal disc viewed from the endothelial side. A, B, Type-1 big bubbles (BBs). The bubble occupies the center of the sclerocorneal disc and are well circumscribed and circular. A, A black circle is drawn to delineate the periphery of the cornea, beyond which is a frill of sclera. C, D, Type-2 BBs. The bubbles extend almost to the periphery of the cornea, represented by the black circle drawn in C. The needle is visible in the stroma. Vision blue dye was applied to the endothelium. E, Mixed-type BB. A horse shoe-shaped type-2 BB is seen along 10 clock-hours (from 8 to 6 o’clock) of the sclerocorneal disc. The peripheral margin of this horse shoe-shaped bubble extends very close to the margin of the cornea. The posterior wall of the horse shoe bubble is elevated above the posterior wall of the central type-1 BB, which is seen in the hollow of the horse shoe. F, Another mixed-type BB where the central type-1 BB is associated with a crescent-shaped, narrower type-2 BB inferiorly. The tip of a swab points to the type-2 BB.
with and without DM was evident (Fig 4A). The DL thickness measured a mean ± standard deviation of 10.15±3.6 μ (range, 6.3–15.83 μ) and the DM measured a mean ± standard deviation 10.97±2.36 μ (range, 7.8–13.98 μ). The DL was made of 5 to 8 thin lamellae of tightly packed collagen bundles running in longitudinal, transverse, and oblique directions (Fig 4D). In comparison, the corresponding width of the corneal stroma anterior to DL in uninflated control eyes demonstrated only 3 to 5 lamellae. The fibrils measured 21.70±2.43 nm in DL and 24.20±2.68 nm in the corneal stroma (P<0.001). The interfibrillar distance, measured between centers of adjacent fibrils, was similar in the DL (9.64±7.74 nm) and posterior corneal stroma (10.09±7.91 nm).

Long-spaced collagen was also seen frequently in the DL adjacent to the DM (Fig 4E). On SEM, the collagen bundles at the anterior surface of the DL were more regularly arranged and were parallel to each other (Figs 5A and 6, available at http://aaojournal.org) compared with the stromal bed, which showed a coarse fibrillar crisscross pattern with gaps related to passage of air (Figs 5B and 6). The posterior surface of the DL showed a smooth pleated pattern made of coarse bundles of collagen (Figs 5A and 6). The DM peeled from the DL presented the classical banded and nonbanded zones and endothelial cells (Fig 4C). This confirmed that the DM had not split between banded and nonbanded zones. The anterior surface of the DM was made of fine fibrils and was the smoothest surface examined (Figs 5C and 6).

Strands of collagen bundles could be seen bridging the space between the DL and the stromal bed, especially along the circumference (Figs 4A and 5D). Centrally, ends of broken strands could be seen on the DL and the stromal bed (Figs 4F and 5D). Importantly, unlike the corneal stroma, the DL did not demonstrate any keratocytes. Occasionally, cell debris or a keratocyte was seen in a broken strand or on the most anterior part of the DL (Fig 4F). The edge of the DL was more compact and distinct from the adjacent deep stroma (Figs 5E and 6). The corneal stroma separated by air bubbles revealed fibrils more coarse than those on any other surface examined (Fig 6).

**Immunohistology**

The DL, like corneal stroma, was primarily composed of collagen I (Fig 7A). Collagen V was weakly positive in both DL and stroma. Collagens IV and VI, however, were more positive for DL compared with the corneal stroma, and positivity was denser toward the anterior and posterior surfaces (Fig 7B). The intensity of staining for pro-teoglycans lumican, mimecan, and decorin was similar in the DL and corneal stroma. CD34 was negative in the DL, confirming the lack of keratocytes in DL.

**Discussion**

For a long time, it was considered that the BB technique and others using viscoelastics, and even mechanical maneuvers with instruments, laid bare the DM in the DALK procedure.4-9

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**Figure 2.** A, The Descemet's membrane (DM) is being peeled off the surface of a type-1 big bubble (BB). The fold of the DM is clearly visible, exposing the Dua's layer underneath. The bubble has not deflated. B, A narrow strip of the DM is being peeled off another type-1 BB, which has not deflated.

**Figure 3.** A, Striae induced by tugging, in the Dua's layer (from which the Descemet's membrane [DM] had been removed) extending beyond the edge of the big bubble (BB) on to the adjacent uncleaved surface. B, A fully inflated type-1 BB from which the DM has been removed. Fine radial striae are seen from the edge of the BB toward the periphery of the cornea all along the circumference.
Recent studies of specimens obtained during unsuccessful DALK operations and from eye bank eyes have reported the presence of “residual stroma” on the DM.\textsuperscript{10-12} Another recent study\textsuperscript{13} of eye bank eyes has demonstrated that air injection into the stroma induced separation of the DM with no residual stroma in 14 of 16 corneas attempted. Our study has clearly demonstrated that a BB can be formed by the separation of the DM only or by a cleavage occurring in a plane along the last row of keratocytes separating a thin but tough layer of corneal collagen along with the DM. That the DM could be completely peeled off the type-1 BB without deflating it and that a type-1 BB could be formed after first peeling off the DM indicates that the DL is a distinct layer that is impervious to air. The presence of the

![Figure 4. A. Light photomicrograph of resin section stained with toluidine blue showing a type-1 big bubble (BB) from which the Descemet’s membrane (DM) has been peeled off centrally to reveal the Dua’s layer (DL). At the periphery of the cleavage, the DM can be seen on the posterior surface of the DL. Strands of collagen bundles (S) are seen bridging the stromal bed and posterior surface of the DL. A keratocyte (arrow) is seen in the anterior surface of the DL (objective $\times40$). B–F, Transmission electron micrographs. B, The posterior wall of a type-1 BB showing the DL closely applied to the DM. The DL is seen to be made of multiple thin lamellae. Part of an endothelial cell (EC) is visible on the posterior surface of the DM. Bar = 10 microns. C, The posterior wall of a type-2 BB showing that it is made up of DM and attached EC only. The banded (BZ) and nonbanded (NBZ) zones of the DM are clearly visible. This was exactly the same with the DM peeled off a type-1 BB. No split in the BZ and NBZ was seen in either instance. Bar = 5 microns. D, The DL from a type-1 BB showing multiple lamellae with collagen bundles running in longitudinal, transverse, and oblique directions. The lamellae are compactly arranged. Bar = 5 microns. E, The DL of a type-1 BB from which the DM has not been peeled off. The banded zone of the DM is seen adjacent to the DL with long-spaced collagen (arrow). Bar = 1 micron. This is also evident in (D), where it appears as dark material in the posterior lamellae of the DL. F, The DL from a type-1 BB showing a coiled end of a broken strand on its anterior surface (between the 2 arrows). Some keratocyte cellular debris is also present.](image)
DM is not essential for its formation. This contrasts with a type-2 BB, which deflates immediately as the DM is peeled off from the edge of the cornea to the edge of the bubble. The corneal stroma allows air to be forced in between the lamellae, where numerous tiny bubbles accumulate, rendering it opaque. When air reaches the impervious DL, it affects a cleavage. Even at high pressure, air did not pass through this layer, which held until it burst. Type-2 BBs, on the other hand, were always formed from the periphery toward the center. This suggests that the DL ends before the end of the DM. Air escaping posteriorly beyond the edge of the DL at the periphery of the cornea thus gains access to the plane between the DM and posterior surface of the DL, producing a type-2 BB. Although all our experiments were carried out with air injection, it is anticipated that the same types of bubbles are observed when viscoelastic is used instead because it would be forced along the same planes. The relatively tightly packed lamellae and greater space between fibrils in the DL possibly accommodating a greater amount of proteoglycans, could contribute to making it airtight.

In 2002, Hirano et al. identified a layer attached to the deep stroma removed by mechanical dissection. They attributed this to a split between banded and nonbanded layers of the DM. With their BB technique, Anwar and Teichmann demonstrated that a white, semiopaque, circular ring in the cornea indicated the formation of the BB. Later, they described another less common type of bubble with the clear edge, which they suggested was because of air between the 2 layers of the DM. Jafarinasab et al. in 2010 described “residual stroma” adherent to the DM in samples removed after attempted BB-DALK converted to penetrating keratoplasty. Similarly, McKee et al. created a BB in human sclerocorneal discs by injecting air from the anterior and posterior surfaces. They also demonstrated “residual stroma” attached to the DM in all but 2 cases. Both studies concluded that the BB technique does not bare the DM in most cases, with the split
occurring within the stroma; however, in another study of 14 eye bank eyes, air injection into the periphery produced DM separation with no residual stroma.\textsuperscript{13} The latter investigators had inserted the needle in the peripheral 1 to 2 mm of the cornea, which is very likely to have been peripheral to the termination of the DL, forcing air directly above the peripheral part of the DM, separating it from the DL without affecting a cleavage of the DL itself. These studies complement data presented in this study, which supports our hypothesis that the BB cleaves off a distinct layer at the posterior surface of the corneal stroma, which is not “residual stroma.” Although the cleavage extends only ≤9 mm of the central cornea and is strongly adherent thereafter, the fine wrinkles in the DL extended further to the periphery but not as far as the DM. When air is forced into corneal stroma the affected area becomes white. The “white ring” seen at the edge of the type-1 BB can be explained by air being forced into the stroma at the cleavage plane, rendering it white. This also explains why the type-1 BB is always circular and does not extend to the corneal periphery during DALK surgery. Most DALK procedures carried out by the BB technique are between 7 and 8.5 mm in diameter and partial thickness trephination is carried out before injecting air. The surgeon continues to inject air until the white ring reaches the trephine mark. On the basis of our study, we contend that larger diameter DALKs should not be attempted by the BB technique as the cleavage between DL and posterior stroma is unlikely to extend beyond 8.5 mm. Instances of sudden bursting of the BB during DALK have been experienced by us and other surgeons (unpublished data, Ramesh K, Glasgow, Scotland, June 2009, and Dua HS and Said DG, Nottingham, England, February 2011). Given the toughness (popping pressure) of the DL, it is very likely that BB bursting is a risk in type-2 BB, where the DM is not supported by the DL. It is also the clinical impression of several surgeons that eyes with DALK have stronger wounds than eyes with penetrating keratoplasty. This too can be attributed to the strength of intact DL left behind in the former.

With lamellar keratoplasty, the occurrence of interface haze remains an issue. Clinical experience has taught us that DALK and ultrathin Descemet’s stripping endothelial keratoplasty are associated with less interface problems compared with anterior lamellar keratopasty and Descemet’s stripping endothelial keratoplasty.\textsuperscript{16–18} Hence, retention of the DL together with the DM and endothelium in DALK is unlikely to be associated with increased risk of interface haze compared with retention of the DM alone. Knowledge of the existence of this layer and its characteristics will influence corneal surgery; for example, the plane between the DL and stroma can be exploited in generating tissue for endothelial transplant, allowing easier handling and insertion of the tissue because it does not tend to scroll as much as the DM, with the DL splitting the DM. It will also help our understanding of posterior corneal pathology such as acute hydrops in keratoconus and pre-Descemet’s dystrophies. The shape and biomechanical properties of the cornea are attributed to the compact anterior lamellae and Bowman zone. We suggest that the tough posterior DL may also contribute in this regard.

Because all experiments were carried out in adult eyes with a mean age of 77.7 years and a median of 82 years, these data cannot be directly extrapolated to younger eyes. However, because the majority of DALK procedures are carried out for keratoconus, wherein the clinical observations supporting the existence of DL are evident, it is reasonable to suggest that the layer is well-defined in that age group too. Further studies are needed to define its characteristics in very young children. We were unable to explore this question as part of this study because of the paucity of children’s eyes that are available for research.

References


Footnotes and Financial Disclosures

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