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Three-dimensional arrangement of elastic fibers in the human corneal stroma☆

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ABSTRACT

The cornea is the main refracting lens in the eye. As part of the outer tunic it has to be resilient, a property conferred by the organisation of the constituent collagen. It also has to be sufficiently elastic to regain its exact shape when deformed, in order not to distort the retinal image. The basis of this elasticity is not fully understood. The purpose of this study was to characterise in three dimensions the arrangement and distribution of elastic fibers in the human corneal stroma, using serial block face scanning electron microscopy. We have demonstrated that there exists a complex network of elastic fibers that appear to originate in the sclera or limbus. These appear as elastic sheets in the limbus and peripheral cornea immediately above the trabecular meshwork which itself appears to extend above Descemet's membrane in the peripheral stroma. From these sheets, elastic fibers extend into the cornea; moving centrally they bifurcate and trifurcate into narrower fibers and are concentrated in the posterior stroma immediately above Descemet's membrane. We contend that elastic sheets will play an important role in the biomechanical deformation and recovery of the peripheral cornea. The network may also have practical implications for understanding the structural basis behind a number of corneal surgeries.

1. Introduction

The optical and biomechanical properties of the human cornea are largely governed by the collagen-rich stroma. This layer constitutes approximately 90% of the total thickness and comprises about two hundred stacked lamellae in the central region. Stromal transparency has long been known to arise from the spatial arrangement of the parallel collagen fibrils within each lamella (Maurice, 1957; Meek et al., 2003), which itself is governed by the association of collagen with interfibrillar proteoglycans (Lewis et al., 2010; Cheng and Pinsky, 2013). The biomechanical properties depend on the arrangement of these lamellae throughout the tissue (Aghamohammadzadeh et al., 2004; Petsche and Pinsky, 2013; Whitford et al., 2015). However, one aspect of the corneal stroma that has been somewhat neglected with respect to corneal development and biomechanics, is the presence of elastic tissue. In fact, elastic tissue was reported in the cornea as far back as 1860 (Kolliker, 1860). In 1906, M'ilroy (1906) described a distribution of elastic fibers found chiefly in the deeper layers of the peripheral human corneal stroma, near Descemet's membrane. Half a century later, Fullmer and Lillie (1958) used the term oxytalan for fibers in periodontal membranes that were resistant to acid hydrolysis. Alexander and Garner (1983) distinguished three types of ocular elastic fibers: oxytalan (precursor fibers without elastin); elaunin (intermediate elastic fibers) and true elastic fibers (with a sheath of glycoproteins surrounding an elastin-rich core). All three types of elastic fiber were present in the mature human sclera, but none were found in the normal adult cornea until 2010, when Kamma-Lorger et al. (2010) reported a network of fibers using two photon fluorescence (TPF) microscopy. These fibers were shown to run roughly parallel to the collagen lamellae in the circumcorneal

Abbreviations: TPF, Two photon fluorescence; DALK, deep anterior lamellar keratoplasty; DSEK, Descemet's stripping endothelial keratoplasty; DMEK, Descemet's membrane endothelial keratoplasty; TEM, Transmission electron microscopy; SHG, Second harmonic generation; SBF, SEM Serial block face scanning electron microscopy; HDBR, Human Developmental Biology Resource; SEM, scanning electron microscope; PEG, polyethylene glycol; PBS, phosphate buffered saline; IMS, industrial methylated spirit; TM, trabecular meshwork; IOP, intraocular pressure.

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annulus (Newton and Meek, 1998), a deep limbal structure that is supposed to help maintain the change in curvature between the cornea and the sclera (Abahussin et al., 2009; Boote et al., 2009). They were also seen in smaller quantities within the central stroma. Recently, Hanlon et al. (2015), using serial block face scanning electron microscopy, described the presence of microfibril bundles in the murine cornea, which they identified as being rich in the protein fibrillin, an important component of elastic fibers (Rosenbloom et al., 1993).

Recent years have seen the development of a number of surgical techniques (deep anterior lamellar keratoplasty (DALK); Descemet's stripping endothelial keratoplasty (DSEK); Descemet's membrane endothelial keratoplasty (DMEK) etc.) aimed at avoiding penetrating keratoplasty. One DALK procedure (pneumodissection) involves injecting air into the stroma, leading to the formation of a so-called big bubble that allows easier separation of the endothelium and Descemet's membrane from the stroma. During these types of procedure, it was noticed that there were different cleavage planes, sometimes immediately above Descemet's membrane and sometimes slightly higher within the stroma (Jafarinasab et al., 2010; Mckee et al., 2011) and in 2013, Dua et al. (2013) proposed that the corneal stroma adjacent to Descemet's membrane was a distinct layer, which has different biomechanical properties to the rest of the stroma. The presence of a distinct new layer is controversial (Jester et al., 2013; Mckee et al., 2014) and it has been suggested that the plane above this so-called pre-Descemet's stromal layer is non-reproducibly determined by the variable distances of keratocytes to Descemet's membrane within and between corneas (Schlotzer-Schrehardt et al., 2015). Nevertheless these developments have highlighted the need to explore in detail the structural and hence the biomechanical properties of the corneal stroma at all positions and depths, and this must include the elastic fibers as well as the collagen lamellae.

In this paper we use conventional transmission electron microscopy (TEM), serial block face scanning electron microscopy (SBF SEM) and the nonlinear microscopy techniques second harmonic generation (SHG) and two photon fluorescence (TPF), which reveal the three-dimensional organisation of fibrous collagen and elastic fibers, respectively. Combining these techniques allows comparison of the structures seen at low magnifications (Green et al., 2014) with those seen at the high magnification electron microscope level, within the human corneal stroma.

2. Methods

2.1. Tissue specimens

Five human corneas were obtained from the CTS Eye Bank, Bristol, UK. Cornea 1 was from a 69-year–old female. The whole enucleated eye was fixed in 4% paraformaldehyde. The cornea was removed with a scleral rim and placed in modified Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer at pH 7.2) for 30 min, dissected and processed for serial block face scanning electron microscopy (SBF SEM) and TEM. Cornea 2 was from a 50-year-old male and was received from the Eye Bank in Eagle's minimum essential medium as a cornea with about 2 mm of the adjacent sclera. It was de-swelled with 8% dextran overnight, mounted in a Barron artificial anterior chamber to maintain a trans-corneal pressure and was fixed using the same modified Karnovsky's fixative for 3 h. It was then dissected and used for SBF SEM and TEM. Cornea 3 was from a 79-year-old male. It was collected and fixed in modified Karnovsky's fixative within two days of death and was processed for TEM using the orcein method (see below). Cornea 4 was from a 72-year old female. It was fixed in 4% paraformaldehyde then wax embedded for histology. Cornea 5 from a 67-year-old male was processed for non-linear microscopy as described below. Cornea 6 was from a 13-week old foetus obtained from the Human Developmental Biology Resource (HDBR). The whole globe was fixed in modified Karnovsky's fixative and was then processed for SBF SEM as below. Institutional Ethics Committee approval was obtained for this study and the research followed the Tenets of the Declaration of Helsinki.

2.2. Serial block face scanning electron microscopy

Human Corneas were fixed for 3 h in 2.5% glutaraldehyde/2% paraformaldehyde in 100 mM sodium cacodylate buffer pH 7.2 at room temperature (RT). The cornea was cut into thin segments and post fixed with 1% osmium tetroxide for 1 h. After a brief wash with fresh buffer followed by a 20 min wash in distilled water the samples were incubated in 0.5% low molecular weight (di-Gallic) $C_{14}H_{10}O_9$ tannic acid (mw 322.22) (AR Mallinckrodt, Dublin, Ireland) in distilled water for 2 h. The samples were then washed in distilled water for 30 min and placed in 1% aqueous uranyl acetate for 1 h in the dark at RT. Corneal samples were then dehydrated in an ethanol series from 70% to 100% ethanol for 1 h after which they were placed in 2% uranyl acetate in 100% ethanol for 2 h in the dark at RT. After washing in 100% ethanol for 40 min, the corneal samples were placed in a 1:1 mixture of 100% ethanol: 100% acetone for 20 min. The samples were then incubated in a saturated solution of lead acetate in a mixture of 1:1 100% ethanol 100% acetone for 2 h (Kushida, 1966). After staining, the corneal samples were washed in two changes of 1:1 mixture of 100% ethanol and 100% acetone for 15 min and placed in 100% solution of acetone for 20 min. After washing the samples 3 times for 20 min in 100% acetone, the samples were finally embedded in CY212 (TAAB) epoxy resin and polymerised for 24 h at 60° C.

The surfaces of polymerised resin blocks were then trimmed and attached to Gatan (PEP6590) specimen pins. The pins were then gold coated and transferred to a Zeiss Sigma VP FEG SEM equipped with a Gatan 3View2 system, where data sets of up to 1000 images were acquired of the block surface every 50 nm through automated sectioning. Each image was acquired at 4K × 4K pixels, at a pixel resolution of 4 nm and a pixel dwell time of 8 μs, using an accelerating voltage of 3.4 keV in low vacuum variable pressure mode (28 Pa). Imaging data was acquired from a 16.19 μm × 16.19 μm region of interest. Selected serial image sequences were extracted from the image data and 3D reconstructions were generated with Amira 6.0 software (FEI, Mérignac, France) using both manual hand tracing for the larger structures and automated thresholding for the fibers.

For the full thickness quantification analysis, the peripheral cornea was imaged every 50 nm en face at 5000 × magnification, starting from the epithelium and ending at Descemet's membrane, obtaining over 21,000 images with resolutions of 2000 × 2000 pixels. The data were split into sets of ~1000 images, fibers were segmented using a mixture of manual and automatic thresholding, resulting in a fiber voxel count that was subsequently converted to percentage using the total volume voxel count. Cells were blocked out to background level in order to remove their contribution to the images. The more detailed quantification above Descemet's membrane was carried out by measuring fiber volume every 200 images. Both were done using Amira 6.0 software with XImagePAQ extension. When sectioning the 16 μm × 16 μm block face through a thickness of almost 1 mm, it was very difficult to ensure that the en face sections were exactly parallel to the surface of the cornea. When viewing our series of images from different depths, it was evident that the blocks were being cut at a slight angle to the surface of the cornea. To correct for this, the number of 50 nm sections between where Descemet's membrane was just visible