The stroma and keratoconus: a review

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Introduction

The cornea is the transparent anterior portion of the fibrous coat of the eye¹. In humans the cornea averages 0.52 mm in thickness centrally thickening to 0.65 mm in the periphery²⁻⁵. The human cornea consists of five layers: the epithelium, Bowman's membrane, the stroma, Descemets's membrane and the endothelium²⁻⁵. The stroma makes up approximately 90% of the thickness of the cornea and is the major structural component. The cornea's strength, shape and transparency can be attributed to the anatomic and metabolic properties of the stroma^{3, 4}. The stroma consists of collagen, glycosaminoglycans, keratocytes and nerves. Two to three percent of the stroma consists of cellular components (keratocytes). Collagen makes up approximately 70% of the dry weight of the cornea. Type I collagen makes up the majority of the collagen in the stroma with types III, IV and VI also being present⁴.

Keratoconus is a developmental or dystrophic deformity of the cornea in which it becomes cone-shaped due to thinning and stretching of the tissue in its central area¹. Depending on the diagnostic criteria used, keratoconus has a prevalence between 50 and 230 people per 100 000.⁴ Keratoconus occurs in all races and there is no gender preference⁴. An association between connective tissue disease and keratoconus has been suggested⁴. Connective tissue diseases like Ehlers-Danlos syndrome, Marfans' syndrome, osteogenesis imperfecta, Reiger's syndrome, among others, have been associated with keratoconus^{4, 6}.

Stroma

The stroma consists of many (hundreds) bundles of parallel collagen fibrils that collectively are called lamellae. Each fibril in the lamellae is highly uniform in diameter being 22.5-35 nm with each fibril running from limbus to limbus. Lamellae vary in size and they divide, rejoin and interweave. The distance between each collagen fibril in the lamellae is also highly regular being between 41.4 \pm 0.5 nm and 60 nm.^{3, 4} The regular arrangement of the fibrils within the stroma is thought to be an important prerequisite for the transparency of the cornea⁴. Each collagen fibril has a dark band crossing the fibril at regular intervals, the periodicity of such banding being approximately 65 nm.^{3, 4} It is thought that the banding of the fibrils indicates that the collagen is embryonic collagen.

An important component of the corneal stroma is the extra-cellular matrix made up of glycosaminoglycans and collagen. Collagen will be discussed further later in this article. Insoluble carbohydrate polymers serve as connective tissues in animals⁷. There are three main classes of carbohydrates, monosaccharides, oligosaccharides and polysaccharides^{7, 8}. Monosaccharides consist of a single polyhydroxy aldehyde or ketone unit⁷. Oligosaccharides consist of short chains of monosaccharide units joined by glycosidic bonds. Polysaccharides are polymers that contain at least 20 monosaccharide units and can contain many thousands of such monosaccharide units⁷. Because the extracellular matrix (also known as ground substance) is composed of polysaccharides, a little more attention is going to be given to these. Polysaccharides, also known as glycans, make up most of the carbohydrates found in nature. They differ from each other in terms of the monosaccharide units that are used to build each polysaccharide, the length of their constituent chains, in the types of bonds that hold each link together and in their degree of branching⁷. There are two main types of polysaccharide, homopolysaccharides which contain only one type of monomer, and heteropolysaccharides which contain two or more different kinds of monomer.



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The extracellular matrix is a gel-like material that fills the extracellular spaces in tissue. The extracellular matrix holds cells together and provides a porous pathway for the movement of nutrients and oxygen to the cells. Heteropolysaccharides and fibrous proteins like collagen, elastin and fibronectin form a meshwork that constitutes the extracellular matrix⁷. The heteropolysaccharides that make up the extracellular matrix are known as glycosaminoglycans. One of the monosaccharides that repeat as a unit in the glycosaminoglycan is always either N-acetylglucosamine or N-acetylgalactosamine. The other repeating unit is usually a uronic acid⁷. A number of glycosaminoglycans occur in the cornea: hyaluronic acid, chondroitin, keratin sulphate and heparin being some of them⁹.

Another important, complex structure in the extracellular matrix of the cornea is known as a proteoglycan¹⁰. Proteoglycans are characterized by glycosaminoglycans molecules attached to relatively small protein cores forming a long cable-like protein with a fuzz of glycosaminoglycans along its length^{9, 10}. The glycosaminoglycan component forms the larger portion of the proteoglycan molecule and is often the main site of biological activity⁷. Mammalian cells can produce up to 30 different types of molecule that can be considered members of the proteoglycan super family⁷. These molecules regulate the extracellular arrangement of collagen⁷. In the corneal stroma collagen is embedded in proteoglycans^{9, 10}. Because proteoglycans take up a lot of space and have a strong negative charge (resulting in a strong affinity for positively charged ions like sodium) there is a strong tendency to imbibe considerable amounts of water if left to do so¹⁰. It is for this reason that many of the anatomic and physiologic specializations of the cornea are designed to prevent the imbibition of large amounts of water into the stroma¹⁰.

Collagen and proteoglycans in the cornea are manufactured and maintained by fibroblasts (also known as keratocytes) a specialized type of cell found in all connective tissues¹⁰. Protein content and organization of collagen is regulated by the fibroblasts in mature tissue and they are able to repair collagen fibrils after trauma¹⁰. The fibroblast is the predominant cell in the stroma⁶. It is a large flattened cell with many processes extending outwards from the cell body that can be found packed between the collagen lamellae. The processes usually extend within or between the same lamellar plane with occasional touching of the tips of adjacent cells⁶. Stromal injury results in the fibroblasts moving into the area that has been wounded where they contribute to scar formation and collagen repair⁶.

Collagen

Collagen is the most abundant protein in the human body and it provides a structural backbone to many tissues, the cornea being one of them⁹. Although collagen is a protein, because it has glycosylated peptide chains, it is also a glycoprotein. The structure of proteins at the atomic and molecular level is extremely complex and hence only a superficial discussion of the structure of collagen will be attempted here. Due to certain constraints at the atomic level, the simplest manner in which a polypeptide (components of the protein structure) chain can be arranged is in a helical structure known as the α -helix. The structure of the α -helix consists of a polypeptide chain that is tightly wound around an imaginary central axis that is constructed through the middle of the helix⁷.

Proteins consist of polymers of macromolecules that are composed of L- α -amino acids linked by peptide bonds. Being composed of amino acids, all proteins contain carbon, oxygen and nitrogen with most proteins also containing sulphur⁸. A basic repertoire of twenty amino acids is used to construct most natural proteins^{8, 11}. Some proteins, however, do contain other amino acids and collagen is one of them, containing hydroxyproline and hydroxylysine¹¹. Many protein structures also contain ions, small organic ligands and water molecules. The *conformation* of a polypeptide means the shape of the curve that the backbone of the molecule traces out. In the native state, under normal physiological conditions, all protein molecules that contain the same amino acid sequence have the same conformation (in other words, they have the same shape). The conformation of a protein molecule is essentially determined by the angles of internal rotation that can occur around the bonds that exist between the various components (residues) of the molecule¹¹. Collagen has a unique helix (a helix whose characteristics are determined by the conformation principles discussed above) in that, unlike the typical α -helix which is right-handed, it is left-handed in terms of the spiral of the helix⁷. Collagen is also a coiled-coil meaning that there are three left-handed coils that are braided or plaited together by means of a right-handed spiral forming the coiled-coil⁷. It is these coiled-coils that form the collagen fibrils making the lamellae in the stroma of the cornea. There are at least twelve types



of human collagen (types I, II, III *et cetera*), however, they all have a common basic structure based on the coiled-coil described above. The individual fibrils (the helix) are assembled in different ways depending on what the task is that that specific type of collagen is expected to perform. The different amino acid sequences and compositions determine their modes of assembly and function^{9, 11}. The typical electron microscope photograph of the stroma, showing the collagen lamellae layered at right-angles are the result of the assembly process alluded to above.



Figure 1a Schematic of collagen fibrils with proteoglycan cross-links between fibrils (figures adapted from reference 13).



Figure 1b Schematic of collagen fibrils with proteoglycan cross-links between fibrils looking along the axis of the fibril. Each fibril is connected to the next nearest neighbor collagen.

Collagen in normal corneas

Collagen in the corneal stroma plays an important role in at least two aspects, firstly the microscopic aspect where collagen aspect where it ensures transparency of the cornea and secondly the macroscopic aspect where collagen imparts shape and strength¹². Microscopically collagen fibrils in the stroma consist of many (in the order of 250) collagen molecules that are axially staggered at regular intervals (as stated above the axial periodicity of the collagen is approximately 65 nm)^{3, 4, 12}. Each fibril is thought to either fuse with limbal collagen or continue into the sclera as electron microscopy has failed to detect an end to the fibrils¹⁴. Fibril diameter is highly uniform^{3, 14} with the average diameter remaining constant until there is a sharp increase in diameter as the fibril approaches the limbus¹². When attempting to elucidate the structure of the stroma electron microscopy results in an underestimation of the centre-to-centre spacing of collagen fibrils due to the methods used to prepare specimens¹².



The exact lateral arrangement of the fibrils is also difficult to appreciate when studying electron micrographs. Using improved methods of preparing corneal specimens, Muller *et al*¹³ re-evaluated the structure of the stromal collagen and they have produced some interesting findings. Among the findings of this study are the following: proteoglycans and collagen fibrils form a dense inter-connected network. Each collagen fibril is enclosed along its length in a coating made up of proteoglycans. Each collagen fibril is connected to other fibrils along its length by strands of proteoglycans. The proteoglycans connecting collagen fibrils do not extend between neighboring fibrils but rather next-nearest neighbor. The interconnecting proteoglycans are uniformly spaced along each fibril, being 42 mm apart¹³ (Figures 1a and 1b show the relationships between adjacent fibrils and inter-connecting proteoglycans). These findings possibly improve on Maurice's original suggestions relating to the structure of the cornea. *J Physiol* 1962 **2** 561-572). Macroscopically the stroma has over 300 collagen lamellae that are stacked on top of each other centrally with up 500 lamellae found near the limbus¹². The lamellae are layered in sheets that are oriented at ninety degrees to each other. X-ray scattering has shown that by far the majority of fibrils in the central cornea are oriented around the inferior/superior and nasal/temporal directions¹².

Various types of collagen are known to exist in the corneal stroma^{9, 14} and it has been suggested that the stability of the stromal matrix may depend to some extent on the proportion of the different types of collagen¹⁴. It has also been suggested that an alteration in the proportions of the different types of collagen may result in destabilization of the connective tissue matrix¹⁴. Of the different types of collagen that may be present in the stroma type I is by far the most abundant, making up 80-90% of the total collagen component^{9, 11}. Approximately 10% of the stroma is thought to be made up of type V and 1-2% type III .⁹

Collagen in keratoconus

Making use of synchrotron X-ray diffraction, Fullwood *et al*¹⁵ investigated collagen molecular and fibrillar packing and the arrangement of proteoglycans along the fibrils. Their findings show the following: there is no significant difference in the interfibrillar spacing of keratoconic corneas compared with normal corneas, keratoconic corneas have significantly lower intermolecular spacing which might be due to changes in the cross links that exist between the fibrils and there is uneven reduction in the staining of proteoglycans, a phenomenon that is probably due to abnormal forms of keratan sulfate proteoglycans having reduced staining when stained with cupromeromic blue. Prior to this study there was conflicting evidence relating to molecular changes and proteoglycans in keratoconic corneas. Sawaguichi *et al*¹⁶ showed that lattice-like and sharply edged ruptures occur in Bowman's membrane in keratoconic corneas, a finding that was dependant on the severity of the condition. In severe keratoconus the ruptures were shown to be partially, or totally, filled with hypertrophic collagen.

The organization of collagen fibrils in the cornea is critical to the maintenance of the shape of the cornea and it is accepted that some form of structural change or modification must be present in the collagen fibrils and lamellae of the keratoconic cornea¹⁷⁻¹⁹. Many studies (using electron microscopy and X-ray diffraction techniques) have found that the preferred orientation of collagen fibrils in the normal cornea is along the vertical and horizontal meridians (the fibrils are also found to be orthogonal)^{12, 17-19}. There is also a regular increase in collagen mass from the center of the cornea towards the periphery^{18, 19}. Only approximately 33 percent of collagen fibrils are more than 22 degrees away from the vertical/horizontal orientation¹⁷. In the keratoconic cornea, however, the preferred orientation of the collagen fibrils is no longer along the vertical and horizontal meridians but shifts towards a preferred orientation of 20/160 degrees¹⁷⁻¹⁹. There is also an increase in the irregularity of the scattering of the fibrils¹⁷. The orientation of collagen fibrils inside the cone is altered considerably while those in the periphery of the keratoconic cornea retain the preferred vertical/horizontal orientation¹⁷. The apex of the cone shows the greatest disturbance to the normal fibril arrangement¹⁹ as well as the most severe loss of collagen mass^{18, 19}. An important question that needs to be addressed is why keratoconus corneas develop the changes that have been alluded to above. Is stromal thinning due to an enzyme mediated loss of collagen, a disruption of fibrillogenesis caused by defective keratocytes, abnormal proteoglycans resulting in the degradation of collagen¹⁷ or slippage between the fibrils leading to a redistribution of collagen away from the apex?¹⁹ There is no clear answer at present.



The stroma in keratoconus

Large numbers of keratocytes are found in the human stroma²⁰. Keratocytes are spindle shaped and have long processes that extend horizontally with some keratocytes having extensions vertically through different lamellae (known as translamellar keratocytes)²⁰. Gap junctions exist between individual keratocytes enabling communication across the cornea. Collagen fibrils are produced by the keratocytes during development and are maintained actively during life. Electron microscope studies have shown how collagen fibrils emanate from the keratocyte wall through microscopic pores²⁰. Keratocytes have also been shown to play a role in supporting lamellar stability²⁰. Collagen metabolism (synthesis and degradation) is dependent on keratocytes in the stroma²¹. Keratocytes also produce various matrix metalloproteases that are thought to play a role in the breakdown of the extracellular matrices of the stroma²¹. Keratocytes are reported to produce collagen and collagenase in response to corneal wounding when tissue remodeling takes place as part of the healing process²¹. They also die, by means of apoptosis, in response to wounding²². It appears then that keratocytes play an important role in the production and maintenance of collagen. The presence of keratoconus brings into question whether keratocytes play any role in this progressive disease.

The pathophysiology of keratoconus is not fully understood at present. One possible explanation for the development of keratoconus is increased apoptosis of keratocytes in keratoconic corneas²³. Apoptosis is a form of cell death that results in minimal release of degrading enzymes and other components that could damage surrounding tissues²³. It is also understood to be a type of programmed cell death²⁴. Kim *et al*²³ have shown that there is increased keratocyte apoptosis in keratoconic corneas as compared to corneas with stromal dystrophy or normal corneas. Kim *et al*²³ go on to suggest that the release of cytokines by damaged epithelial cells might be the trigger for keratocyte apoptosis in keratoconus.

Confocal microscopy allows for the non-invasive observation of the various layers of the cornea including keratocytes and they have the specific advantage over conventional microscopes of being able to show structures in the cornea that would normally be difficult due to the transparent nature of corneal tissue²⁵. Numerous studies have been published where confocal microscopes have been used to investigate keratocyte density²⁵, long-term effects of contact lens wear²⁶ and keratocyte density in keratoconic corneas²⁷⁻²⁹. Several confocal microscope studies have shown that the mean keratocyte density in the anterior and posterior stroma is decreased in the keratoconic cornea and that contact lens wearing keratoconic corneas have an even greater decrease in keratocyte density²⁷⁻²⁹. Hollingsworth *et al*²⁸ have also shown a relationship between the amount of haze detected using confocal microscopy and keratocyte density with an increase in haze being related to a decrease in keratocyte density. The decrease in keratocyte density has also been associated with atopy, eye rubbing and corneal staining²⁸.

The fact that matrix metalloproteases²¹ and cytokines²³ play a role in the normal physiology of the stroma has been alluded to above. Matthews *et al*³⁰ have shown that TIMP-3 (tissue inhibitor of matrix metalloproteases) induces stromal cell apoptosis with scarred keratoconic corneas having significantly more apoptotic cells than non-scarred keratoconic corneas. Mackiewicz *et al*³¹ have also produced evidence relating the presence of various matrix metalloproteinases and collagenolytic enzymes to keratoconus.

Collagen crosslinking

More than two decades ago reports were occurring that investigated the existence of crosslinks in collagen³². It was known at that time that collagen is subject to different types of posttranslational modifications, intra- and intermolecular covalent crosslinking being one of them. It was suggested that decreased crosslinking would be present in keratoconic corneas³². Various methods of inducing increased crosslinking between collagen fibrils was investigated by Spoerl *et al*³³ who found that the mechanical stiffness of the cornea can be increased by exposing the corneal stroma to riboflavin and UVA radiation (other methods were also found to be successful). It was suggested that the increased formation of crosslinks could be used in the treatment of keratoconus to prevent the progressive degradation in keratoconus caused by proteolytic activity³³. In more recent studies^{34, 35} it has been shown that riboflavin/ultraviolet exposure does in fact induce a stiffening of keratoconic corneas that seems to have beneficial results to the keratoconic, benefits like: an improvement in best corrected VA, postoperative regression of the mean keratometer values, and postoperative reductions in refractive compensations (by an average of 1.14 ± 2.18 D). In untreated fellow control eyes normal progression of the keratoconic state continued. Of course



exposure to UVA radiation raises the question of the safely of this technique. Wollensak *et al*³⁶ have addressed this issue (at least to some extent) and found that, at the levels of UV exposure used in this technique, there is no endothelial damage caused. The use of riboflavin and UVA exposure in the treatment of keratoconus is new, however, it does offer an alternative to the sufferers of keratoconus, an alternative that could possibly afford a permanent resolution to the progressive nature of this disease. Further research will be needed to determine if crosslinking does provide such a permanent solution.

Conclusion

Keratoconus is an inconvenient condition for any one to have to endure. Its cause is unknown and as optometric clinicians we are limited to the fitting of contact lenses (and rarely spectacles) to keratoconic patients in an effort to provide those patients with adequate sight. Keratoconus is a complicated disease that appears to have at least some foundation at the molecular level of the structure and development of the corneal stroma. There is adequate evidence of the types of changes or aberrations that occur in the keratoconic cornea (this review has presented only a small amount of that evidence) and as our understanding of the types of changes that occur develops and improves so too will our ability to develop interventions that will provide better and more permanent treatments for this debilitating condition.

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