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Perspective

Morphological changes in keratoconus: pathology or pathogenesis

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ABSTRACT

Keratoconus was first discriminated from other corneal ectatic diseases in 1854. Since that time the morphological characteristics of keratoconic progression have been invaluable in the diagnosis of the condition. The key clinical features used to identify keratoconus have remained essentially the same since the introduction of the slit-lamp biomicroscope. Only relatively recently has the development of computerized corneal topography revolutionized the diagnosis of early keratoconus. Analysis of peer-reviewed literature databases revealed a steady chronological increase in pathological research into the progress of keratoconus. This overview describes the recent advances in our understanding of keratoconic pathology and highlights the interactions within the cornea that may be important in the pathogenesis of this condition.

Key words: cornea, diagnosis, keratoconus, pathogenesis, pathology.

INTRODUCTION

The diversity and complexity of keratoconus

Keratoconus is a corneal ectatic disease in which the cornea develops a conical shape, due to thinning of the corneal stroma, with subsequent irregular astigmatism and myopia leading to marked impairment of vision.[1] Extensive research has concentrated on elucidation of the aetiology and disease progression, but due to highly variable phenotype expression and signs these remain a central enigma in ophthalmology and vision science. Indeed, keratoconus typically manifests at puberty and progresses until the third or fourth decade of life, alternatively it may commence later and arrest at any age. Usually an isolated condition it may also coexist with many other disorders, and due to the diversity of clinical presentation, the reported incidence varies widely between 50 and 230 per 100 000 of the general population.[1]

Keratoconus appears to have an unusually high prevalence in New Zealand, with 50% of corneal transplants performed being for keratoconus compared with 30% in Australia[2] and 20% in the UK.[3]

Clinical signs of keratoconus

Since the first adequate description of keratoconus, which set it aside from other ectatic conditions, by Nottingham in his treatise 'Practical observations on conical cornea: and on the short sight, and other defects of vision connected with it'[4] significant advances have been made in the recognition of this condition.

The most recent advances in computerized corneal topographical assessment have made the diagnosis of keratoconus more emphatic. Prior to the widespread availability of this technology diagnosis was more difficult due to highly variable signs of disease progression and the frequent absence of signs in the early stages of the disease. In fact, analysis of the clinical features of keratoconus from three different generations of ophthalmologists highlights how little the diagnosis changed over the intervening period.

In his excellent review of keratoconus in 1998, Rabinowitz lists the following clinical signs that may be present individually, or in combination, in moderate to advanced keratoconus:[1]

> Stromal thinning (centrally or paracentrally, most commonly inferiorly or inferotemporally); conical protrusion; an iron line partially or completely surrounding the cone (Fleischer's ring); and fine vertical lines in the deep stroma and Descemet's membrane (Vogt's striae) … Other accompanying signs might include epithelial nebulae, anterior stromal scars, enlarged corneal nerves and increased intensity of the corneal endothelial reflex and subepithelial fibrillary lines.

The long-established awareness of these clinical signs is confirmed by examination of much older literature on keratoconus.

Indeed Berliner in 1943 listed the seven distinct alterations in keratoconus[5] as classified by Von der Heydt and

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Appelbaum: (i) thinning of the cornea at the apex of the cone; (ii) reflex from the endothelial cup; (iii) striae; (iv) irregular superficial opacities or scars; (v) ruptures in Descemet's membrane; (vi) increased visibility of the nerve fibres; and (vii) Fleischer's ring.

These signs of keratoconus have been cemented into ophthalmological dogma in Duke Elder's 'System of Ophthalmology' in 1965, who noted:[6]

1. A thinning of the cornea at the apex of the cone from one-half to one-fifth of its normal dimensions.
2. An endothelial reflex appears in the central portion of the cornea at the peak of the cone.
3. Vertical lines are seen in the deeper layers of the stroma.
4. An increased visibility of the nerve fibres which form a network of grey lines interspersed with small dots.
5. Fleischer's ring, a line running round the base of the cone.
6. Ruptures of Descemet's membrane of characteristic appearance.
7. Ruptures in Bowman's layer in advanced cases producing superficial linear scars.

Thus the seven key diagnostic features of keratoconus have evolved little in 60 years and interestingly in 1965, Duke-Elder noted that 'pathological investigations have provided little to add to the biomicroscopic (clinical) appearance'.[6] The present review specifically assesses the impact of pathological investigations since that time.

KERATOCONUS RESEARCH

History of keratoconus research

The availability of research information, via the plethora of medical science databases through the Internet, allowed analysis of the research foci in the field of keratoconus since Duke-Elder's critical appraisal in 1965.[6]

Databases were searched in November 2003 and Ovid Medline was chosen as the key database. 'Keratoconus' as a keyword identified a total of 1927 research papers citing keratoconus within the title, abstract, keywords or MeSH headings. When keratoconus was mapped to subject headings, it revealed 'Keratoconus' as a major subject heading in 1612 publications. This latter total should therefore represent publications in which keratoconus forms the major interest of the manuscript. The subheadings within the major subject heading of keratoconus were analysed and are shown in Fig. 1. Of the total of 1612 publications cited within the database, with keratoconus as a major subject heading, 375 (23.3%) were in the subspecialty of pathology.

Pathology forms the backbone of keratoconus research

The publication history in the field of keratoconus was analysed in 5-year groups since the seminal work of Duke-Elder in 1965. Database searches were performed using 'keratoconus' as a major subject heading for each 5-year period and then by limiting each 5-year search period to those publications in the subspecialty of pathology. The results of this database analysis are shown in Fig. 2.

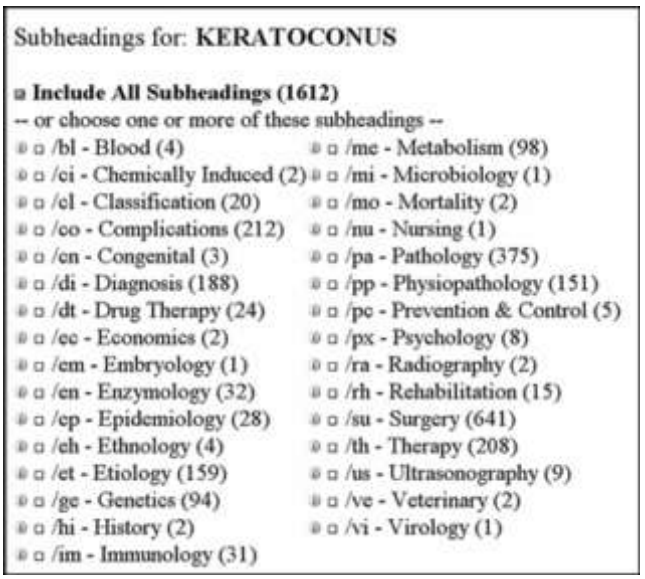

Figure 1. Edited screen grab from an Ovid Medline literature search using the main subject heading 'keratoconus', revealing a total of 1612 publications mapping to that heading. The subheadings are listed and in brackets the number of publications that map to that discipline within the area of keratoconus research.

The number of publications in the field of keratoconus has increased from 106 per 5-year period in the 1960s to approximately 250 per 5-year period in the last 10 years. Although this increase per se is significant, it does not keep pace with the increase in the number of biomedical journals and the subsequent exponential rise in published papers. This may reflect researchers growing frustration with the complexity of the keratoconic disease process. However, pathology papers published in the field of keratoconus increased from 20% in the 1960s to a peak of 34% in the late 1990s. Pathology therefore represents an increasingly important subspecialty area in keratoconus research that may provide insights into the progression of this enigmatic disease.

MORPHOLOGICAL CHANGES IN KERATOCONUS

Histopathological abnormalities have been documented in every layer of the keratoconic cornea. The following represents a layer by layer summary of morphological variations reported within the last 15 years. The results are summarised in Fig. 3.

Epithelium

Ex vivo histological analysis of keratoconic corneas has identified significant thinning of the central epithelium.

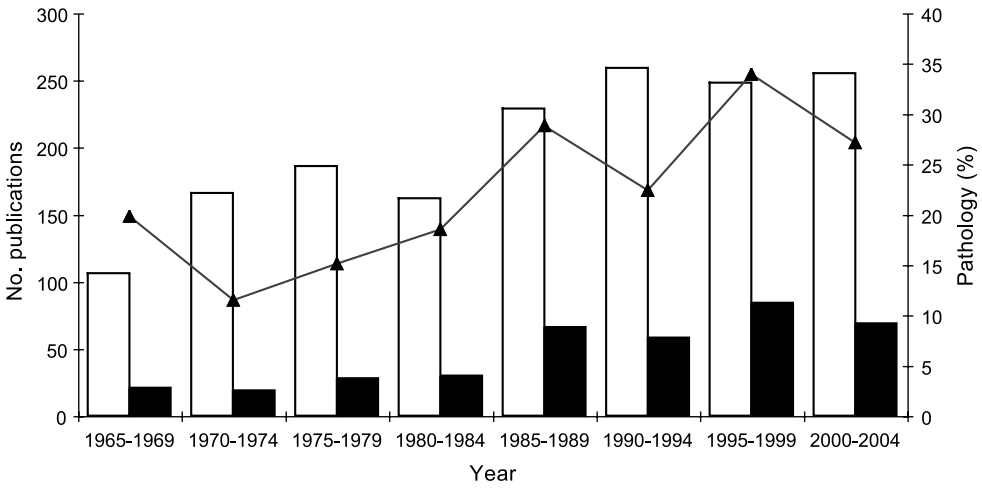

Figure 2. Graphical analysis of the number of publications on the subject of keratoconus broken down into 5-year intervals since 1965. (□) All keratoconus; (■) keratoconus pathology; (▲) percentage of the overall keratoconus publication rate that pathology represents.

Scroggs *et al.* demonstrated that the central epithelium was thinned in explanted corneas.[7] These authors described two groups of keratoconic corneas, 'typical' and 'atypical', with typical including corneas with breaks in Bowman's layer (see later section) and atypical being devoid of these breaks. The central epithelial thinning was significantly greater in the typical group; however, the authors thought it unlikely that these histopathological variations represented different pathogenic mechanisms. The variable nature of keratoconus is highlighted by contrasting reports of keratoconic epithelia being severely thickened,[8,9] whereas another study found no difference in thickness between control and keratoconic epithelium.[10]

In vivo confocal microscopy studies of the epithelium demonstrate that although normal epithelial cells can be found in the periphery of keratoconic corneas, the superficial epithelial cells located at the apex of the cone are extremely elongated and arranged in a whorl-like fashion. The apex of the cone also contains highly reflective structures and fold-like changes in the basal cell layer.[11] These *in vivo* changes may well reflect disruptions of the basal epithelium integrity in keratoconus. Apoptotic changes have been detected in epithelia of keratoconic samples. One study determined that TUNEL-positive epithelial cells were only detectable in the superficial epithelium of normal corneas whereas in keratoconic corneas many TUNEL-positive epithelial cells were detected at lower levels in the epithelium.[8] This is supported by the work of Kaldawy *et al.*[12] who reported that intense TUNEL labelling was present in the basal epithelia of 15 of 16 keratoconic corneas. The authors further confirmed this with the apoptosis specific ssDNA stain, finding staining evident in the epithelium.

Basement membrane

The keratoconic basement membrane assumes an irregular appearance and localized breaks.[13] Tuori *et al.* examined the immunohistochemical composition of the basement membrane in detail and found varying results.[14] Laminin-1 and laminin-5 staining was shown to be irregular and thickened at defect sites; however, monoclonal antibodies against the α2 and β2 chains did not react. Type IV collagen α1 and α2 reactivity was also only found in the defect regions of keratoconic or scarred corneas. Immunostaining for type VII collagen was patchily localized to the basement membrane defects. Integrin β4 staining, which was positive in the basement membrane and the lateral and apical cell membranes of the epithelial cells, was found to be discontinuous in keratoconic corneas. The authors concluded that scarring alone was not responsible for the changes in the basement membrane in keratoconus and suggested that a process similar to wound healing would account for the differences found in keratoconic corneas.

Cheng *et al.* noted that the staining intensity for type XII collagen was reduced in the epithelial basement membrane zone and stromal matrices in keratoconic corneas.[15] They suggested that these alterations may affect critical interactions of the corneal epithelium with the basement membrane, cell–matrix interactions and matrix organization in the stroma.

Nerve fibres

The increased visibility of nerve fibres by slit-lamp biomicroscopy in keratoconic corneas *in vivo* has become a central tenet in ophthalmology. However, examination of the available literature reveals little information as to why this should occur. Recently, our laboratory has conducted studies into the involvement of nerve fibres in the progression of keratoconus.[16] In this study we were able to identify nerve fibre thickening in the sub basal plexus layer using a probe that labels an intra-axonal protein. These nerve thickenings were found in close association with deformities in Bowman's layer and keratocytes were identified in intimate association with the nerve fibres as they encroached upon this normally acellular layer. These nerve thickenings and the close association of keratocytes might explain the enhanced visibility of 'nerve fibres' in keratoconus.

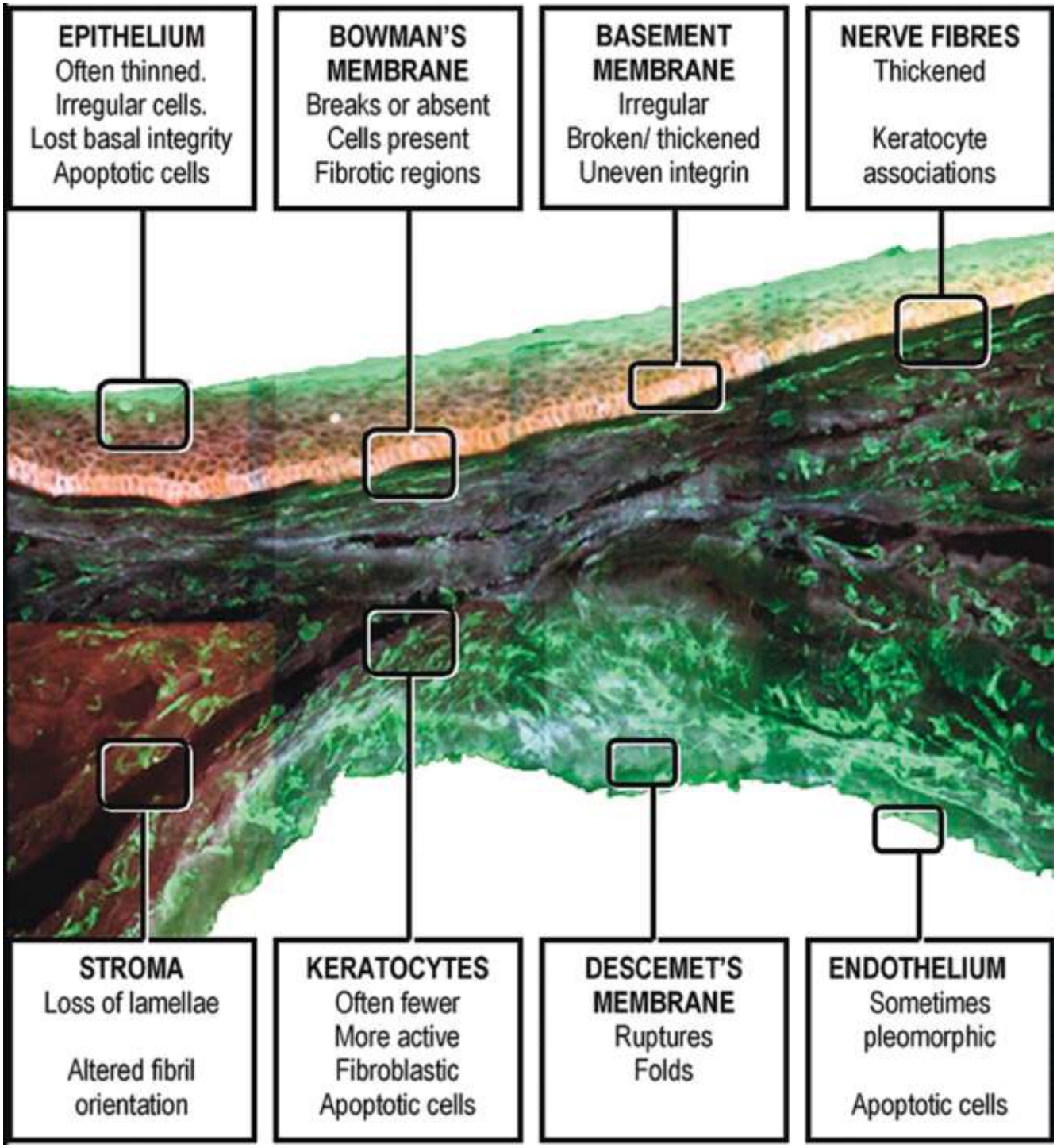

Figure 3. An anteroposterior section of the central 1 mm of a keratoconic cone from penetrating keratoplasty surgery. The tissue has been labelled with CellTracker Green (Molecular Probes) to mark viable cells and then counter-stained with antibodies to integrin (red) and fibronectin (blue). The cross-section shows some of the classical features of keratoconic pathology. Areas of the cornea are highlighted to show position and type of pathological features in keratoconus. Reprinted from Sherwin *et al.*,[9] with permission from Elsevier.

Bowman's layer

Structural abnormalities and defects in Bowman's layer in the central part of keratoconic cornea have been well documented. Sawaguchi *et al.* examined the collagen network in keratoconic tissue by scanning electron microscopy and found sharply edged defects and ruptures in Bowman's layer to varying degrees in all corneae examined.[17] Tuori *et al.* noted discontinuities in Bowman's layer and, occasionally, distorted stroma beneath these defects.[14] Kenney *et al.* noted

gaps in Bowman's layer and also observed fibrotic regions where the epithelium was in direct contact with the stroma.[18] In addition, these authors observed that abnormalities of the extracellular matrix were not uniform within an individual keratoconic cornea, again suggesting localized areas of disease progression.

Most studies of keratoconus tend to target the central cone of the cornea for analysis, as this is usually the area of greatest disease expression. Our studies have targeted the peripheral keratoconic cone in the hope of identifying early pathological features.[9] We noted that peripheral keratoconic corneae exhibited discrete incursion of fine cellular processes into Bowman's layer. These processes originated from keratocytes and were often observed in conjunction with a defined indentation from the basal epithelium.

Stroma

Collagen lamellae

Transmission electron microscopy studies of diseased tissue have revealed that the thickness of collagen lamellae is unaltered in keratoconus, but the number of lamellae appears to be significantly less than in normal tissue.[19] Synchrotron X-ray diffraction studies indicate no difference in interfibrillar spacing between keratoconus and control corneas, thus unambiguously demonstrating that thinning of the corneal stroma in keratoconus is not a result of closer packing of the fibrils in the corneal stroma. However, some evidence is presented for a reduction in the volume of proteoglycan between the collagen fibrils in keratoconic cornea.[20] This suggests progressive loss of lamellae from the stroma but how this loss is initiated and the fate of the affected collagen and keratocytes is unknown. Low angle X-ray scattering has shown that the orientation of collagen fibrils within the lamellae is also altered in keratoconus,[21] suggesting that loss of structural integrity, degradation and/or insufficient repair mechanisms may all be important in the disease process. Biochemical analyses of the stromal matrix components have not clarified the matrix changes as the literature describing collagen levels in keratoconic corneae is inconclusive: Critchfield *et al.* described decreased collagen and total protein levels in keratoconic tissue by Western blotting.[22] Radda *et al.* found a 5% increase in type I collagen in keratoconus;[23] and Zimmermann *et al.* found no differences in collagen composition of biochemical extracts from keratoconus.[24]

Keratocytes

Laser scanning fluorescence microscopy studies have revealed changes in keratocyte morphology in keratoconic corneas.[11] *In vivo* confocal microscope studies have examined the keratocyte density in keratoconic corneas.[10] These authors noted that keratocyte density was 12% lower in all of the keratoconus corneas when compared with normal corneas. The loss of keratocytes could be due to apoptosis, which has been detected in keratocytes in 60% of keratoconic corneas in one study[8] and 11 out of 16 corneas (68%) in another.[12] The first of these authors posed the question: 'If keratocyte apoptosis contributes to the pathogenesis of keratoconus, then why weren't apoptotic keratocytes detected in 100% of keratoconic corneas?' Two possible explanations were offered. First, apoptotic keratocytes might not be detected during a period of keratoconic remission. Second, and very importantly, they propose that 'keratoconus is diagnosed on the basis of clinical findings … There may be several diseases with differing pathophysiological mechanisms that produce the phenotypic change that is referred to as keratoconus'.

The *in vivo* analysis of keratocyte density performed by Erie *et al.* went on to analyse keratocyte densities at different depths in the keratoconic cornea, finding keratocyte densities lowest in the 0–10% stromal layer (anterior most), the 67–90% and the 91–100% layer (posterior most).[11]

Although there may be a significant decrease in the density of keratocytes in the stroma immediately underneath Bowman's layer, the remaining keratocytes are far from inactive. High voltage electron micrographs digitized using 3-D software revealed breaks in Bowman's layer with keratocytes and their pseudopodia orientated apically towards the break and the overlying epithelium. The activated state of the keratocytes was indicated by the abundance of rough endoplasmic reticulum within the cells.[25] Recently, our laboratory examined areas away from the central cone where the cornea was relatively intact and identified discrete cellular abnormalities in the peripheral cone of keratoconus[9] with discrete keratocyte processes in Bowman's layer and subsequent posterior collapse of epithelial cells into Bowman's layer.

Descemet's membrane

Ruptures and folds in Descemet's membrane are a common feature in keratoconus.[13] The origin of these ruptures is unclear as several studies of extracellular matrix proteins revealed no differences in detection of types I, III, IV, V, VI, or VIII collagen and the same is true of laminin, entactin and perlecan.[18,24] One study noted that although the immunoreactivity was identical in normal, scarred and keratoconic corneas, the reaction was discontinuous within the defects in the keratoconic cones.[14] The appearance of the defects in Descemet's membrane may well be associated with environmental factors such as eye rubbing.

Endothelium

Rabinowitz reports that the endothelium is usually normal in appearance, but some abnormalities including intracellular dark structures, pleomorphism and elongation of cells have been reported.[1] Scanning slit confocal microscopy and ultrasound biomicroscopy in living patients with

keratoconus has revealed central detachment of Descemet's membrane and the endothelium from the posterior part of the stroma.[26] Scanning electron microscopy suggests that endothelial cell loss may be directly due to ruptures in Descemet's membrane as severe degradation of endothelial cells, including cell membrane perforation, loss of cell contents and oedema, was associated with Descemet's rupture.[27] Alternatively endothelial cell loss may be associated with apoptosis, which in one study was identified in the endothelium of 13 of 16 corneas.[12]

Epithelial/keratocyte/nerve interactions

It is clear from the published research that abnormalities in Bowman's layer and the direct interaction of keratocytes and epithelium are a recurring feature of the pathology of keratoconus. The role of nerve fibres in facilitating that interaction remains to be fully elucidated. The enzymatic changes in the keratoconic cornea that accompany these cellular changes are well documented and are succinctly summarized in a recent review.[28] These changes will not be discussed in detail here, but by implication, cellular mechanisms must be responsible at least in part for the structural degradation and pathological changes that accompany advancing keratoconus.

Influence of cytokines

Epithelial–stromal interactions across Bowman's layer are critical in development, homeostasis and wound healing in the cornea.[29] These interactions are mediated by soluble cytokines.

Keratocytes produce hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF), which regulate proliferation, motility and differentiation of epithelial cells.[29] Conversely, epithelial interleukin-1α (IL-1α) appears to be a master regulator of matrix metalloproteinase, HGF and KGF production by keratocytes. Platelet-derived growth factor (PDGF) is reported as a potential 'master' regulator, modulating keratocyte proliferation, chemotaxis and differentiation.[30–34]

To date only one study of cytokine production in keratoconic cornea has been performed and this study showed that lower IL-1α mRNA levels, and slightly higher levels of PDGF mRNA, were present in keratoconic cornea compared to other diseased corneal tissue.[35] However, this difference was not statistically significant. Limitations in respect to these data include that the study examined tissue from surgery, representing the final phase of the disease rather than the pathogenesis, and the total RNA extracts of whole corneal trephines were examined by semiquantitative reverse transcriptase-PCR amplification. Localized cytokine mRNA concentration differences may therefore have been swamped within the analysis of the whole tissue.

The prolific disruptions in Bowman's layer and the close association seen between epithelial cells and keratocytes in keratoconus suggest that the normal cytokine cascade of the anterior cornea must be disrupted in the diseased cornea. Whether this plays a part in the pathogenesis or merely compounds the pathology of keratoconus remains to be elucidated. However, Wilson *et al.* hypothesized that:[36]

> Many other disorders of the cornea are probably influenced by or rooted in acquired or genetic abnormalities of cellular communication. More research is needed to elucidate paths of cellular communication in the cornea and to explore the role of cell–cell interactions in the pathophysiology of corneal disease.

Evidence from recurrence of keratoconus

The role of corneal cells in the pathogenesis of keratoconus is supported by the published reports of recurrence of keratoconus in eyes after penetrating keratoplasty[37] with report of breaks in epithelial basement membrane, disruptions in Bowman's layer, stromal thinning and abnormal keratocytes. Two possible explanations have been put forward:[38] either it is a recurrence of the host's disease in the graft or it is transmission of undiagnosed keratoconus from the donor cornea. A recent study described histological examination of 12 corneal buttons from consecutive patients undergoing repeated penetrating keratoplasty 10–28 years after the initial graft for keratoconus.[39] Although the reason for regraft was corneal ectasia in only three of the cases, the study revealed structural changes compatible with a diagnosis of keratoconus in all 12 buttons. The authors concluded that that recurrence of keratoconus characteristics may result from graft repopulation by the recipient cells, ageing of the grafted tissue, or both.

Conclusion

Pathological studies of keratoconus are slowly elucidating the mechanisms behind the progression of the disease. A diverse range of morphological changes have been described in every layer of the keratoconic cornea. The question remains as to whether the description of the histopathological changes of keratoconus has enlightened the pathogenesis of the disease. It is the authors' belief that further clarification of the pathology of keratoconus will ultimately lead to elucidation of the pathogenesis. The diversity of pathology described in keratoconus is likely to represent temporal differences in the progression of the disease, positional differences relative to the apical centre of maximum damage and possibly reflect a variety of pathophysiological diseases that make up the clinical phenotype we identify as keratoconus. Certainly, future laboratory and clinical studies need to be carefully designed and coordinated to enable the diverse clinical and pathological findings to be further correlated. In this regard the *in vivo* confocal microscope may play an invaluable role in providing the clinic to laboratory link.

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