Eye (2014) 28, 189–195

www.nature.com/eye

CAMBRIDGE OPHTHALMOLOGICAL SYMPOSIUM

The pathogenesis of keratoconus

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Abstract

Keratoconus (KC) is a common degenerative condition that frequently results in visual loss with an onset typically in early adulthood. It is the single most common reason for keratoplasty in the developed world. The cause and underlying pathological mechanism are unknown, but both environmental and genetic factors are thought to contribute to the development of the disease. Various strategies have been employed to address the gap in our understanding of this complex disease, with the expectation that over time more sophisticated therapies will be developed. In this review we summarise our current knowledge of the aetiology and risk factors associated with KC.
Eye (2014) **28,** 189–195; doi:10.1038/eye.2013.278; published online 20 December 2013

Keywords: keratoconus; cornea; molecular genetics

Introduction

Keratoconus (KC, OMIM 14830) is bilateral progressive thinning and ectasia of the cornea that results in blurred vision from irregular astigmatism. The changes may be markedly asymmetric and in advanced disease axial corneal scarring can develop that further reduces vision. A number of different phenotypes are recognised such as predominant axial corneal thinning (apex cone), inferior corneal thinning (dropped cone, pellucid marginal degeneration), or generalised corneal thinning (keratoglobus), but it is unclear whether these are variants of KC or distinct conditions.[1,2] Similarly, it is not known whether a generalised thinning of the cornea without ectasia, or regular astigmatism are risk factors for the development of KC, a partial expression of the disease, or unrelated phenomena.

After the onset of KC various environmental effects such as inflammation, degeneration or scarring cause secondary changes that modify the corneal tissue. Each step of this process has been examined with regard to the pathogenesis of KC, but as with many complex diseases, distinguishing between association, cause and effect is problematic and extremely challenging.[3] Thus, despite a great deal of research, the aetiology of KC is still poorly understood. For KC the currently recognised environmental influences include contact lens wear, chronic eye rubbing and allergic eye disease.[2] Importantly, although the development of disease involves complex interactions between genetic and environmental factors,[4] their relative contributions to disease are currently unknown and likely to be variable. Research strategies to unravel KC pathogenesis include histochemistry, biomechanics, enzymology, proteomics, and molecular genetics. These studies have certainly enhanced our understanding, but are confounded by the fact that research efforts have largely focussed on patients with relatively advanced disease, where it can be difficult to distinguish primary disease mechanisms from secondary inflammatory or degenerative effects, and by the possibility that the clinical appearance of KC can include phenocopies that derive from a number of unrelated environmental or genetic effects. KC may thus be the final common pathway for many different pathological processes. Here, we review the data generated to dissect the pathogenesis of KC in an effort to reconcile the knowledge gained so far.

Structure and proteomics

One of the first theories for the pathogenesis of KC was that the primary insult was an epithelial abnormality, resulting in the release of proteolytic enzymes that degrade stromal collagen to thin and weaken the cornea.[5] Involvement of epithelial cells in the disease process is supported by the observation of structural irregularities in the epithelium

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Received: 18 October 2013
Accepted: 20 November 2013
Published online: 20 December 2013

(blebbing and degeneration) that appear to progress with the disease.[6] However, with light microscopy there is additional evidence of major structural changes in other corneal layers, including breaks in Bowman's layer and stromal thinning.[5,7] The minor changes of cellular pleomorphism of the endothelium are most likely secondary to contact lens induced hypoxia or mechanical stress.[8]

There is conflicting evidence regarding biochemical abnormalities in KC, with reports of increased,[9] decreased,[10] or normal levels[11] of proteoglycans. Examination of the proteome of KC tissue has confirmed that, compared with normal cornea, there is upregulation of decorin and keratocan.[12–14] Keratan sulphate proteoglycan appears to be structurally altered in KC corneas, perhaps containing fewer keratan sulphate chains.[15] The majority of studies agree that in KC the stromal collagen content is decreased[9,10] but that there are no dramatic changes in either the distribution of the various collagen types[16–18] or in the cross-linking pattern[19] and molecular arrangement of the collagen within the stroma.[9,20] The exception is the altered distribution of collagen type III in scarred regions of KC tissue.[18]

Several studies have investigated the KC-associated proteome. Examination of the tears or corneal tissues from patients with KC has identified differential expression of proteins compared with controls. These studies provide additional clues about the pathology of the disease as well as identifying potential targets for treatment. The expression of a range of proteins, including cytokines and enzymes, is altered in KC when compared with normal controls, suggesting the involvement of apoptosis and scarring in the disease process. However, it is unclear whether the pathways are modified as a primary or secondary phenomenon. Examination of tear fluid indicated that there are >1500 proteins in the tears,[21] and that inflammation has a role in the pathogenesis of KC.[22,23] Proteomic studies of the KC epithelial and stromal layers have demonstrated that structural remodelling and metabolic stress occur in both layers. Many extracellular matrix proteoglycans and proteins involved in proliferation, growth and migration were found to be downregulated in KC epithelium and stroma, and oxidative stress was evident.[24,25]

Biomechanics

The corneal tissue in KC is less rigid than normal tissue[26] and an increased axial length suggests a relationship with myopia.[27] The stroma is predominantly formed of water and type I collagen. The collagen fibrils lie parallel to each other within lamellae, which are themselves stacked parallel to the surface of the cornea. Stromal thinning, a hallmark of KC, is caused by a reduction in the number of lamellae within the affected region[28] rather than compaction of collagen fibrils within individual lamellae,[29] but the mechanism by which the thinning occurs is uncertain. Although stromal thinning in KC has been attributed to collagen degradation by proteolytic enzymes[30,31] or decreased levels of proteinase inhibitors,[32] it has also been proposed that collagen is not lost but simply redistributed within the cornea by slippage between the lamellae.[33] This latter mechanism is supported by the observation of reduced inter-lamellar adhesion,[34] lamellar interlacing in the apex of KC corneas[35,36] and a reduced number of lamellar insertions into Bowman's layer.[35] X-ray diffraction studies provide some of the strongest evidence to support this theory. In the deeper stromal layers of the healthy cornea the collagen is predominantly aligned in the superior–inferior and nasal–temporal orthogonal directions.[37,38] However, this arrangement was markedly altered in the apical region of advanced KC.[19,39,40] These later studies also presented evidence of a redistribution of lamellae away from the apex of the cone and highlight a variability of collagen distribution patterns between individual cases.[19,39] On the basis of these findings, Meek *et al* proposed that the loss of structural integrity in the KC cornea was caused by the presence of abnormal keratocytes and matrix proteins[15,31] and upregulated proteolysis triggered an unravelling of lamellae along their length and from their anchors at the limbus, with an opening of the lamellar bifurcations. This theory is supported by observations following riboflavin/UVA collagen cross-linking, where the proposed cross-linkage of the tissue increases both the resistance of the stroma to enzymatic digestion and the cohesiveness between collagen fibrils and the non-collagenous matrix.[41] The molecular basis of the abnormality in the matrix collagen interaction has yet to be determined.

Genetics

The reported prevalence of KC is highly variable ranging from 2340 per 100 000 in Israel[42] to 0.2–0.4 per 100 000 in Russia.[43] This large discrepancy may in part reflect differences in diagnostic criteria and the age group studied, however such variability is also likely to reflect the differences in genetic variation in such populations. In the UK, prevalence of KC has been estimated to be 4.4–7.5 times greater for Asians compared with white Caucasians.[44,45] Although the majority of cases of KC are sporadic, reports of familial cases of KC are also common and aggregation of the disease in families is often observed.[46] The prevalence of KC in first degree relatives of index cases has been estimated to be 3.34%, notably higher than that in the general population.[47]

Concordance is also high among monozygotic twins and a greater similarity of phenotype is observed in monozygotic twin pairs implying that genetic factors are likely to have a key role in the disease phenotype.[48]

KC has been associated with a wide range of systemic and ocular conditions, for example Leber congenital amaurosis,[49] anterior polar cataract,[50] Brittle cornea syndrome,[51,52] and with a 10–300-fold higher prevalence in individuals with Down syndrome.[53,54] Interestingly, individuals with connective tissue disorders such as Ehlers Danlos syndrome have a higher prevalence of KC.[55,56] The caveat to these observations is that mutations in the causative genes have not been shown to be enriched or causative in isolated KC cases. Therefore, the relevance of the increased occurrence of KC in these genetic conditions has yet to be established. Genetic risk factors for KC have proven difficult to identify because of the complex nature of the condition. Several genetic approaches have been employed, including linkage studies in families with predominantly suspected dominant inheritance of KC, candidate gene analysis in KC cohorts, and genome-wide association studies (GWAS) to identify risk loci. Candidate genes for KC have been considered primarily on the basis of their biological function. For example, the superoxide dismutase isoenzyme 1 encoded by *SOD1* located on chromosome 21 was considered an attractive candidate gene because oxidative stress is hypothesised to have a role in the aetiology of KC[57] and there is an increased prevalence of KC in patients with Down syndrome (trisomy 21).[58] Despite the identification of an intronic deletion that segregated with KC in two small families,[58] this finding has not been replicated in additional cohorts,[59,60] and it remains to be established whether variants in this gene are associated with KC. Similarly, other studies have investigated the transcription factor visual system homeobox 1 encoded by *VSX1*, a gene implicated in posterior polymorphous corneal dystrophy (PPCD), because these PPCD patients have localised steepening of the anterior cornea similar to KC.[61] To date, more than 1500 KC patients have been screened for *VSX1* and potentially pathogenic mutations have been identified in less than 2% of cases suggesting that this gene does not have a major role in the molecular pathology of KC.[60,62–64] Other candidate genes screened in KC cohorts include *TIMP3*,[65] *TGFBI*,[66,67] *ZEB1*,[68] *FLG*[69] and several collagen genes.[46,70,71] Despite these efforts, potentially pathogenic variants have only been identified in a very small number of individuals with KC.

Although large families with KC are very uncommon, several groups have described extended familial inheritance of KC, the majority with an apparent dominant, or dominant partially penetrant inheritance pattern. In these familial cases, linkage analysis has been performed. This approach has proven to be powerful for other familial forms of complex disease such as myopia[72] and systemic lupus erythematosus.[73] To date, 17 distinct potential loci for KC are described;[74] however, only three of these loci have been independently replicated, 5q21,[75,76] 5q32[75,77] and 14q11.[75,77] Collectively, these findings suggest that there is a high degree of genetic heterogeneity for KC. Unfortunately, convincing KC-associated genes have not been identified within these reported loci, with one notable exception; the identification of a potentially pathogenic variant in candidate gene *DOCK9*, segregating in a large dominant KC family of Ecuadorian origin.[78] There are several reasons for this lack of identification of causative genes from linkage studies. Using conventional linkage strategies to understand complex diseases relies on the assumption that the condition is caused by a variant that has a large effect, and this may be an over-interpretation of familial aggregation of KC in many instances.[79] The application of linkage analysis is also complicated by the possibility of phenocopies and reduced penetrance in any given KC pedigree. The inclusion of individuals with simple astigmatism, thin corneas without ectasia, or borderline (*forme fruste*) KC disease is also controversial. In addition, despite the typical age of KC onset in the second decade of life, onset has been reported over the age of 50 years[80] making the classification of individuals as unaffected difficult. Furthermore, large families are required to obtain robust linkage and many of the studies performed to date have not identified regions of linkage with significant LOD scores.[74] Finally, different loci and genes may be implicated in families of different ethnicities, confounding replication of the loci. Given these complexities, there is hope that progress will be made using recent advances in next-generation sequencing technologies. Time- and cost-effective strategies to sequence all genes in the defined locus, or the entire linked region, for genetic variants is now a realistic possibility and will undoubtedly facilitate progress to identify the genetic variant(s) segregating with disease in these extended families.[81] Of particular relevance, linkage analysis in combination with next-generation sequencing identified a heterozygous mutation in mir-184 in two families with dominant congenital cataract associated with a corneal phenotype that in some cases was consistent with KC.[50,82,83] To establish whether mutations in mir-184 were associated with KC, mir-184 was subsequently screened in a cohort of 780 KC patients.[84] Rare variants were identified in two (0.25%) patients, but the variants did not fully segregate with disease, suggesting that mir-184 variants are not a common cause of isolated KC.[84]

Genome-wide association studies (GWAS) are a powerful tool to identify common variants of relatively

low effect as risk factors for complex disease.[85] Several KC GWAS have been performed, but with relatively small numbers of patients. An early study that included three cohorts from Australia, USA and Northern Ireland implicated genetic variation at the *HGF* locus with KC susceptibility, although it did not reach genome-wide significance.[86] A more recent KC GWAS case–control study for a Caucasian cohort from the USA identified a single-nucleotide polymorphism (SNP) near *RAB3GAP1* as a potential susceptibility locus for KC.[87] Again the study did not reach genome-wide significance, but this finding has been replicated in an Australian Caucasian cohort.[88]

Another approach is to test for association with endophenotypes of the disease, rather than a case–control study, or to combine these approaches. There has been much interest in defining loci associated with central corneal thickness (CCT), which is a highly heritable quantitative trait.[89] Reduced CCT is a hallmark of KC and a risk factor for primary open-angle glaucoma.[90] Recently, an elegant and extensive study described a meta-analysis of GWAS for CCT that included over 20 000 Caucasian and Asian individuals. This study identified 16 new loci associated with CCT at genome-wide significance.[91] To investigate whether any CCT-associated loci identified also influence genetic susceptibility to KC, the authors tested for association of these loci in a case–control study including 874 patients with KC. This meta-analysis identified six SNPs that were strongly associated with a risk of KC, within or nearby the following genes/loci; *FOXO1*, *FNDC3B*, *RXRA-COL5A1*, *MPDZ-NF1B*, *COL5A1*, and *ZNF469*. Of the six significant loci, the SNP rs9938149 ~160 kb upstream of *ZNF469* is interesting as bi-allelic mutations in this gene cause Brittle cornea syndrome in which patients have extremely thin and fragile corneas;[51] however, GWAS showed an unexpected effect direction, with the CCT-increasing allele leading to an increased risk for KC. The authors concluded that these findings demonstrated that part of the genetic predisposition to KC is mediated through genes underlying CCT, with the remaining predisposition attributable to alternative mechanisms.[91] This conclusion mirrors the clinical finding that the occurrence of a relatively thin cornea in isolation is distinct from the progressive regional thinning of the cornea that is a feature of KC.

Gene expression studies

Differences between the expression of genes in normal corneas and corneas with KC complement the proteomic studies to identify disease pathways. In addition to many focussed studies on particular genes or gene families, other studies have investigated the transcriptome. Microarray analysis of the KC epithelium compared with controls revealed 56 genes with differential expression, further delineated into genes with a functional role in the cytoskeleton, extracellular matrix, transmembrane signalling, cell–cell interaction, and cell–matrix interaction. The *KRT6* gene was the most upregulated gene in this study.[92] Another study used cultured keratocytes and a specific microarray for apoptosis genes, and concluded that the differentially expressed apoptosis genes identified may have an important role in stromal thinning.[93] Cultured keratocytes with a fibroblast morphology were also used in a genome-wide transcriptome microarray study, revealing a 212-fold reduction in the mRNA levels of alcohol dehydrogenase (class 1) beta polypeptide (ADH1B).[94] However, it is currently not clear if ADH1B levels are a marker for, or a potential mediator of, keratoconus. Cultured corneal stromal fibroblasts from normal and KC corneas were used for a PCR based study, that revealed differential expression of eight genes, including *TIMP1*, *TIMP3* and *BMP4*.[95] *AQP5* which encodes a water channel expressed in the corneal epithelium has been observed to be downregulated in KC corneas compared with controls,[96] however this finding was not replicated by a subsequent study comparing *AQP5* levels in KC and healthy corneas.[97] One recent study used pooled patient corneas for microarray transcriptome analysis, and identified 87 differentially expressed genes.[98] The majority were downregulated, controlled by the transcription factor AP-1.

None of the differentially expressed genes in any of these studies significantly overlap each other, nor do they help validate any of the candidate genes from genetic studies. This is likely due to a combination of the different tissue and cell types used, genetic heterogeneity and the different experimental methods and arrays. They do, however highlight similar pathways of cellular differentiation, proliferation, and apoptosis that correlate with the proteomic data. Such expression analyses are a valuable addition to the other approaches to elucidate the pathogenesis of KC, however further detailed studies are required to tease out primary from secondary effects.

Conclusions

Different approaches have been used to investigate and define the phenotype, mechanisms and causes of KC. Observations of corneal changes that occur in KC often do not distinguish between primary changes and secondary inflammatory or degenerative effects. Although many differences have been identified that distinguish the KC cornea from the normal cornea, it has not been possible to trace these changes back to primary causes, or to identify the triggers that precipitate the

cascade of events that leads to the clinical picture of KC.[99] Similarly, a range of suggestive genetic loci and variants have been implicated in the genetic susceptibility of KC, however the current lack of large effect contributions of these loci, SNPs and gene variants suggests a complex aetiology or convergence of multiple disease pathways. Extrapolating these data to disease mechanisms is currently problematic but the recent advances in next-generation sequencing technologies promise rapid progress in this field.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank the Lanvern Foundation, Moorfields Special Trustees and Moorfields Eye Charity, the National Institute for Health Research (NIHR) (Moorfields Eye Hospital and UCL Institute of Ophthalmology (London, UK)), and Rosetrees Trust for financial support. This is a brief review and by no means comprehensive, and we acknowledge that many important studies are not referenced.

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