2 Modern Pathogenesis of Keratoconus: Genomics and Proteomics

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2.1 Introduction

Despite numerous intensive studies, the pathogenesis of keratoconus remains unknown. Eye rubbing and the presence of allergies seem to be the only commonly identified factors.

The majority of keratoconus cases are temporally sporadic and some forms are defined by the existence of several clinically affected patients within the same family. Autosomal dominant (with reduced penetrance) and autosomal recessive transmission modes have been described [1]. Several studies suggest the existence of subclinical forms within the relatives of an affected patient with keratoconus [2, 3]. Identical twins show strong concordance of keratoconus with a high degree of phenotypic similarities, suggesting a key role for a genetic component [4].

Extreme variations in the prevalence of keratoconus are observed in relation to ethnicity. One study investigating its prevalence in Asian and Caucasian individuals living in similar geographic zones demonstrated an unequal distribution of the disease. Its incidence is four times higher among Asian individuals compared to Caucasians [5]. Others studies report differences in the severity and evolution of the disease in relation to ethnicity, which provides another strong argument in favour of a genetic component [6, 7]. While the multifactorial origin is accepted, the genetic component undoubtedly has a major role [8, 9].

Two pathophysiological mechanisms, probably interrelated, have been proposed: a biomechanical change or a biological origin. The biological origin of the disease can be investigated based on either a candidate hypothesis or a comparative analysis without candidate.

2.2 Candidate-Driven Approach

2.2.1 Candidate Gene Approach

The candidate gene approach is based on the knowledge of the disease biochemistry and pathology and consists of identifying mutations in encoding genes for the proteins of the affected tissue. Several studies found enzymatic and biochemical anomalies in affected corneas [9, 10]. The association of keratoconus with osteogenesis imperfecta [11] and mitral valve disease [12, 13] indicates a potential role for collagen anomalies in its occurrence. Studies investigating the pathogenesis of

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keratoconus have attempted to identify mutations in genes coding for components of interleukin-I [14], proteases [15, 16], protease inhibitors [17, 18], and collagens. Type I, III, IV, V, VI, VII, and VIII collagens are present in the cornea. The first collagen-encoding gene tested was COL6A1, but no significant relationship with keratoconus was detected [19]. Similarly, other collagen-encoding genes of the cornea were tested and eliminated [10]. Negative results obtained from linkage analyses do not exclude the role of these encoding genes in certain types of keratoconus. Indeed, the degree of genetic heterogeneity of this disease is unknown and mutations of several encoding genes from families that have not been tested may yet play a role in the emergence of keratoconus. One candidate gene study led to the identification of related mutations among patients affected by keratoconus. Mutations affecting the gene VSX1 were identified. This gene codes for a putative transcription factor and is also involved in posterior polymorphous dystrophy [20]. VSX1, however, has a role in only 0.1–0.4 % of familial keratoconus and thus its importance in the pathogenesis of keratoconus remains low.

2.2.2 Syndromic Keratoconus

Keratoconus is occasionally associated with other genetic or ophthalmologic diseases such as Down's syndrome, Leber congenital amaurosis, atopic diseases, conjunctivitis, some pigmentary retinopathies, mitral valve prolapse, collagen vascular diseases, and Marfan's syndrome [9, 10, 21]. It remains difficult, however, to establish a direct relationship between these diseases and keratoconus. Interpretations of these relationships are indeed challenging because of the relatively high prevalence of keratoconus, which remains probably underestimated. As a result, it is difficult to determine if these diseases are associated as cofactors triggering a keratoconus genetic susceptibility or if they are directly involved in the disease pathogenesis. This is an issue of particular significance when considering associated genetic diseases that are the results of the alteration of known genes and where a genetic analysis would help identify the gene responsible for keratoconus.

Down's syndrome is strongly related with keratoconus, with an estimated prevalence of 0.5–15 %, which is 10–300 times higher than the general population [10, 22, 23]. This suggests a link between keratoconus and chromosome 21. Numerous genetic studies have targeted this chromosome and some of them suggest its involvement in the pathogenesis of the disease [19]. This relationship, however, has also been attributed to some environmental factors such as eye rubbing, which may play a co-factor role, resulting in a strong prevalence of keratoconus [23].

Among all associated diseases, the direct cause of keratoconus is largely unknown and remains a matter of continued debate. This is predominantly due to the lack of basic information regarding the real prevalence of keratoconus and the impact of environmental factors.

2.2.3 Non-candidate-Driven Approach

An interesting global approach using 'omic' techniques aims to identify the origin of keratoconus without taking into account any preconceived ideas of the pathogenesis of the disease. In this approach, corneas affected by keratoconus are compared with healthy corneas during a particular cell machinery stage: the DNA (genomics), the RNA (transcriptomics), or the protein (proteomics). Transcriptomics is used to analyse, at the mRNA level, which genes are being expressed and in what ratios, whereas proteomics looks at the proteins that are subsequently translated.

2.2.4 Linkage Analysis

Unlike studies investigating candidate genes, genetic linkage analyses do not rely on any knowledge of the pathogenesis or biochemistry of the disease. Linkage analyses highlight genetic regions that contain genetic variants bound to a phenotype.

To date, 17 distinctive genetic regions have been identified [8], indicating the existence of a

strong genetic heterogeneity in the development of keratoconus. Among these regions, only three have been independently verified (5q21, 5q32, and 14q11). These studies implicate two genes as potential minor candidates (*MIR184* and DOCK9). More recently, global sequencing of the genome and/or exome has replaced linkage analyses.

2.2.5 Genome-wide Association Studies

The relative failure of previous approaches and the recent advancements in molecular biology have promoted new studies. Using the state-of-the-art technologies of high-throughput genotyping, recent studies have compared the frequency of hundreds of thousands of genetic variants distributed among chromosomes. The most convincing studies detected linkage with variants of the gene *LOX* (lysyl oxidase, involved in the cross-linking of stromal collagen) [24, 25]. Other authors have concentrated on comparing intermediary phenotypes rather than groups of individuals (case–control study). Lu et al., for example, identified a region associating keratoconus with central corneal thinning [26]. Complementary meta-analyses have identified several variants in or near this region (*FOXO1*, *FNDC3B*, *RXRA-COL5A1*, *MPDZ-NFIB*, *COL5A1*, and *BANP-ZNF469*). One of these variants, *BANP-ZNF469*, was also found in an independent Australian cohort [27]. The role played by variants of this particular gene was confirmed in a study by Lechner et al. [28]. *ZNF469* is a gene that is also involved in another corneal syndrome (brittle cornea syndrome) and is linked to a thin and fragile cornea. This gene is currently the most important identified genetic factor in the pathogenesis of keratoconus.

2.2.6 Transcriptomics–Proteomics

Several studies have compared proteomes between patients affected by keratoconus and control patients. Comparability of results is difficult, due to differences in tissue type (tear fluid, corneal tissue: epithelium and/or stroma), age, disease severity, and development stage of keratoconus. A number of studies provide evidence that keratoconus is characterized by a cytokine imbalance in tear fluid and that these inflammatory mediators operate actively at the ocular surface. More than 1500 proteins have been identified in the analysis of the tear film. Higher levels of proteolytic activity and increased levels of proinflammatory cytokines, cell adhesion molecules, matrix metalloproteinases (MMP), glycoproteins, and transporter proteins have been observed compared to controls [29–32]. In particular, studies have shown a strong concordance of elevated Interleukin 6 (IL6), tumour necrosis factor α (TNFα), and MMP9 in tears from keratoconus patients [29, 33–35]. MMP9 is one of the matrix-degrading enzymes produced by the human corneal epithelium and regulated by cytokine IL6. In addition to IL6, TNFα is considered a major pathogenic factor in systemic and corneal inflammation. TNFα induces the expression of MMP9. Balasubramanian et al. [29] found higher levels of MMP9 in tears from keratoconic eyes, but the difference was not statistically significant. They indicated that the observed discrepancy might be explained because they used antibodies to the active MMP9. Shetty et al. [35] observed that MMP9, IL6, and TNFα were strongly upregulated at the mRNA level in keratoconus patient epithelia. However, whereas tears of keratoconus patients demonstrated an acute increase in MMP9 and IL6 levels over controls, TNFα levels did not show any significant associations with different grades of keratoconus. They demonstrated that the administration of cyclosporine A strongly reduced the inflammatory stimulation and expression of MMP9 in tears of keratoconus patients and decreased the production of IL6, TNFα, and MMP9 by corneal epithelial cells, while follow-up of 20 keratoconus patients over 6 months demonstrated significant local topographical changes in the cornea measured by keratometry. Although overall change in keratometry may not be very significant in the study, the authors suggest that cyclosporine A may be a promising new treatment modality for keratoconus [35].

We should take into account that the reported changes in tear film cytokine profile may not necessarily reflect intracorneal processes in keratoconus.

It is however becoming clear that mediators of inflammation are present in the keratoconus cornea. Keratoconus could be an inflammatory disorder as many studies are indicating elevated levels of inflammatory markers but with contradictory findings. Differences in the expression of some proteins (matrix components, cytokeratin, etc.) have also been observed at both the epithelial and stromal levels [35–37]. Findings overlap only partially, however, bringing into question not only the different pathophysiological routes involved but also the sampling procedures. Although keratoconus is not caused by corneal inflammation itself—clinical and histological findings show little evidence of this inflammation—data strongly substantiate the emerging concept of underlying inflammatory pathways in the pathogenesis of keratoconus. We must also bear in mind that there is the possibility that the changes in these inflammatory mediators may be an epiphenomenon of change in corneal structure.

Overall, it cannot be ruled out that keratoconus originates in events which take place outside the cornea but which are ultimately responsible for the induction of its ectasia. Numerous proteases, immunoglobulins, and cytokines have been found in tear fluid of patients but could reflect changes in lacrimal gland and conjunctiva.

Are these changes cause or effect and are they genetic or environmental in origin? The source of these proteins remains unknown. Atopy, eye rubbing, contact lens wear, oxidative stress [33, 38, 39], and genetic factors have been suggested to cause the disease. Balasubramanian et al. [40] made an interesting observation that eye rubbing increased inflammatory cytokines and MMP levels in tears in normal eyes and in keratoconus.

In addition to local activation of inflammatory pathways, there is accumulating evidence that systemic inflammatory changes [41] and systemic oxidative stress [33, 38, 39] may affect the corneal microenvironment in keratoconus. The interaction of corneal and systemic cellular inflammatory mediators that contribute to development of keratoconus is poorly understood.

Apoptosis of keratocytes is found as well [42, 43]. Based on an RNA study, Mace et al. demonstrated that keratoconus might be related to a deregulation of the proliferation pathways and cellular differentiation [43]. Inadequate balance between cytokines (pro- and anti-inflammatory) may lead to an altered corneal structure and function, triggering an increase in metalloproteinases and keratocytes apoptosis. The exact underlying molecular mechanisms remain to be elucidated.

These studies have shown that keratoconus demonstrates a corneal structural imbalance, associated with a metabolic stress, and an imbalance between apoptosis and proliferation. To date, however, these studies have failed to identify a clinically usable biomarker to screen keratoconus or assess its degree of severity.

2.3 Conclusion

The pathogenesis of keratoconus remains a mystery. Global analyses are beginning to highlight important affected pathways, but considerably more effort is required to understand the development of this disease. Scientific evidence has shown that keratoconus is a multifactorial disease involving complex interaction of both genetic and environmental factors.

Genetic susceptibility is now considered a key factor in the occurrence of keratoconus, but despite extensive studies, no contributing gene has yet been identified. Finding a gene responsible for keratoconus is crucial, as it would allow for the characterization of diagnostic criteria by comparing phenotypes and genotypes. This would aid surgeons as keratoconus is a contraindication to corneal refractive surgery, but it would also help to elucidate the pathogenesis of this disease.

Compliance with Ethical Requirements Pierre Fournié, Stéphane D. Galiacy, and François Malecaze declare that they have no conflict of interest. No human or animal studies were carried out by the authors for this chapter.

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