The role of TNF-α, TGF-β2 and NF-κB in the pathogenesis of keratoconus

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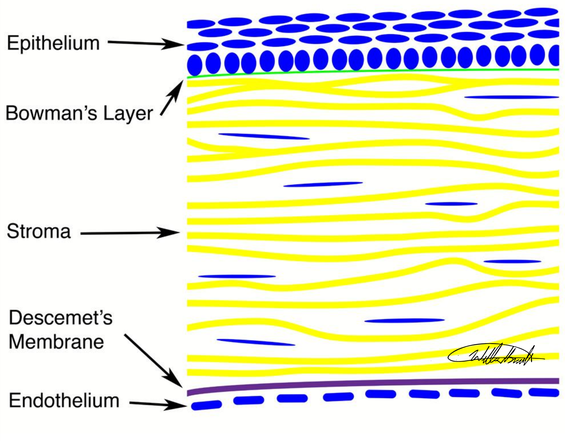
Background

Keratoconus is a non-inflammatory, bilateral, progressive, often asymmetric primary ectasia associated with irregular astigmatism and decreased visual acuity affecting 1 in every 2000 Americans (1,2). It is one of the most common indications for corneal transplantation. Keratoconus usually presents in the second decade of life and progresses into the third and fourth decades affecting both genders and all ethnicities. Previous studies have demonstrated an increased protease activity in the cornea, which could theoretically break down some of the collagen cross-linkages and thus reduce the mechanical strength and stiffness of the cornea (5). A genetic predisposition toward the development of keratoconus has also been demonstrated, which sometimes run in families and identical twins and also in individuals with Down syndrome, Ehlers-Danlos syndrome, and Marfan syndrome (6,7). Keratoconus has also been associated with eye rubbing and atopic diseases such as asthma, allergies and eczema (8). The disease results in progressive dissolution of Bowman membrane. Cellular and structural changes in the cornea affect the integrity and lead to protrusion and scarring (9). Earlier studies suggest a role of oxidative stress and keratocyte apoptosis in the pathogenesis of keratoconus (13-16). Recent investigations have shown an increased expression of the secreted frizzled-related protein-1 (SFRP-1), SFRP-1 and microtubule-associated protein light chain, and transforming growth factor-β (TGFβ) signaling pathway activation and increased TGFβ pathway markers in severe keratoconus (17,18,20). TGF-β is a signal protein which regulates many cellular functions including cell proliferation, differentiation, migration, and survival as well as development, carcinogenesis, fibrosis, wound healing and the immune response (21). TGF-β2 transmits its signal through SMAD3 transcription factor-dependent and independent pathways (22). It was also reported TGF-β activates NF-κB and RhoA, and RhoA activates NF-κB in several kinds of cells in a Smad-independent pathway (22). The use of TGFβ receptor kinase inhibitors or angiotensin pathway blockade increases Bowman membrane integrity in chronic allograft nephropathy (23). TGFβ1 is a known potent chemotactic cytokine to initiate inflammation, but the autoimmune phenotype seen in TGFβ1 knockout mice reversed the concept of it being a pro-inflammatory cytokine to predominantly an immune suppressor (25). The discovery of the role of TGFβ family of genes in Th17 cell activation once again revealed the pro-inflammatory effect of TGFβ1. Keratoconus was previously thought of to be a non-inflammatory condition, however, the presence of TGFβ2 in the keratoconic corneas has inspired the investigations of this project. Activation of both Th1/Th17 cells and regulatory T cells (Tregs) by TGFβ1 reversely regulated by IL-6 shows the dual role of TGFβ1 in regulating inflammation (24). Although not in context with the pathogenesis of keratoconus, recent reports have indicated that IKKγ facilitates RhoA activation, which in turn activates ROCK, leading to direct phosphorylation of IKKβand subsequent activation of NF-κB. Conclusively, the RhoA and IKK complexes may regulate each other and form a positive feedback loop to activate NF-κB (25) Corneal keratocytes (corneal fibroblasts) are specialized fibroblasts that resides in the stroma. This layer of cornea represents about 90% of corneal thickness and is comprised of highly regular collagenous lamellae and extracellular matrix components. Following an injury to the cornea, some keratocytes undergo apoptosis, as a result of expression of the signaling molecules secreted by the upper layers including IL-1α and TNF-α (26).

Normal Cornea Histology



Materials and Methods

Under an Institutional Review Board (IRB) approved protocol, surgically discarded and/or de-identified normal donor and other keratoconic cornea. Keratoconic and normal donor corneas were obtained from the department of Ophthalmology, Loyola University Health System. A total of 12 normal and 12 keratoconic corneas were selected for this study. The tissues samples were fixed in formalin/PIPES buffer and stored at 4C until paraffin embedding and sectioning at 4 microns. Deconvolution immunofluorescence (IF) was performed on the unstained slides. After imaging, relative expression (normal vs keratoconus) of TNF-α, TGFβ2, and NF-κB was quantified in Imaris®. For TNF-α, TGFβ2, and NF-κB, Center channel measurements were obtained per 10 µm cubes (see figures and 1 and 2) in the epithelial and stromal layers of both keratoconic and normal donor corneas. Samples not containing intact epithelium were not quantified. Statistical analysis was performed using Graph Pad Prism one-way ANOVA with multiple comparisons. To determine the intensity of NF-κB in the nucleus, surfaces were created around Imaris® in the 435 nm channel (dapi stained nuclei) and the mean of Cy5 intensity was quantified within those volumes (see figures 3 and 4). Student's unpaired t-test with Welch's correction was performed in graph pad prism. See below images for examples of how Imaris was used to quantify data.

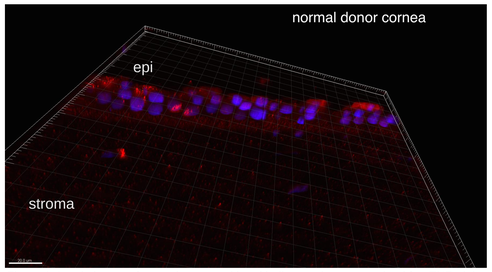


Figure 1: Imaris image of normal cornea with epithelium and stroma stained for TNF-alpha

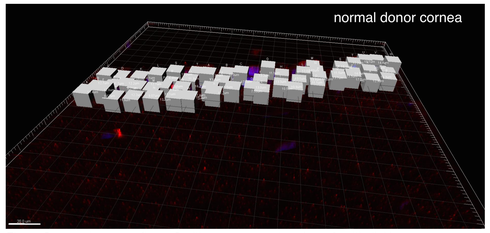


Figure 2: same image as figure 2 with measurements cubes of epithelial layer

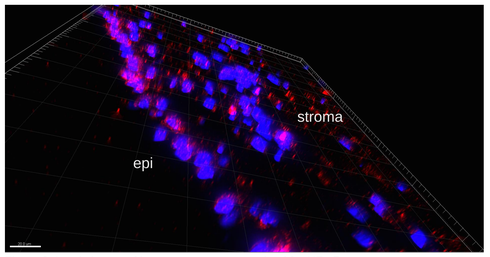


Figure 3: Imaris image of keratoconus cornea stained for NF-κB

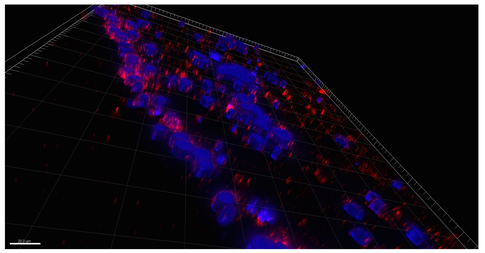


Figure 4: same image as figure 3 volumes created around nuclei, inside which NF-κB intensity was quantified

Results

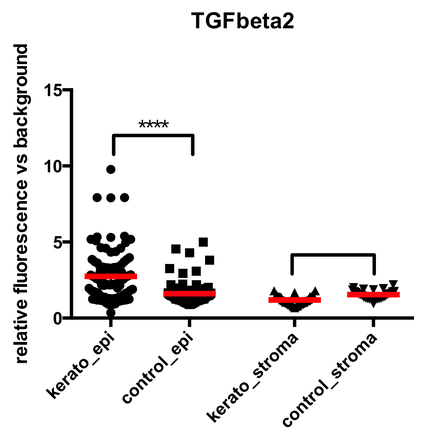


Figure 5: quantification of relative fluorescence (mean signal:noise ratio). TGF-β2 is expressed 1.7 times higher in keratoconus epithelium when compared to normal corneas (p<0.0001)

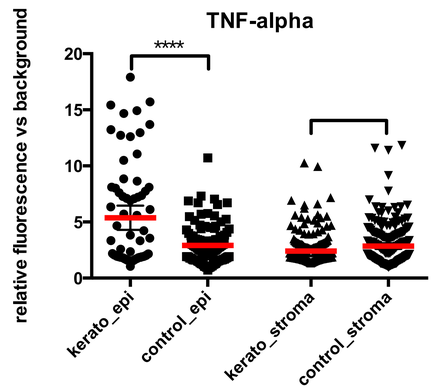


Figure 6: quantification of relative fluorescence (mean signal:noise ratio). TNF-α is expressed at 1.9 times in keratoconus epithelium when compared to normal corneas (p<0.0001).

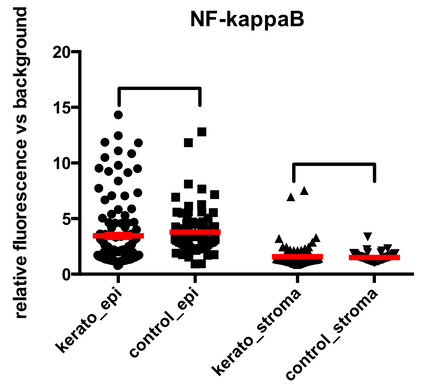


Figure 7: quantification of relative fluorescence (mean signal:noise ratio). NF-κB is expressed 0.9 times as high in keratoconus epithelium when compared to normal corneas (p=0.64).

Results

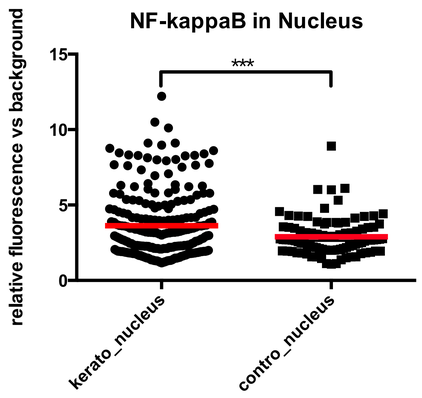


Figure 8: quantification of relative fluorescence (mean signal:noise ratio). NF-signal intensity was 1.3 times higher in keratoconus epithelium when compared to normal corneas (p=0.0002)

Analysis, Conclusion, and Future Experimentation

✧TGF-β2 and TNF-α both demonstrated higher expression in the epithelial layer of keratoconic corneas when compared to the normal corneas (p<0.0001). NF-κB had similar expression levels in the epithelial and stromal layers of keratoconic corneas when compared to normal corneas (p=0.64 and p=0.99 respectively). However, there was higher intensity staining of NF-κB in the nuclei of keratoconic corneal samples when compared to normal corneal samples (p=0.002). These data indicate a paradigm shift in the understanding of keratoconus from non-inflammatory condition to in fact an inflammatory state.

✧Immunofluorescence staining for IL-33 was also performed and there was a very low level of antibody binding (data not shown). This may be due to the short half life of IL-33 and the lag time between surgical resection and tissue fixation. We will consider using fluorescent in situ hybridization with molecular probes with complimentary sequences to IL-33 mRNA.

✧Future directions: Results from an assay that will measure TGF-β2 expression by cultured human corneal endothelial cells (HCEC) after challenge with TNF-α and/or mechanical disruption in the presence or absence of a Smad3 small molecule, SIS3, inhibitor are pending.

Funding and Acknowledgment

Thanks to the Illinois Society for the prevention of Blindness for grant funding and Monika Valtink, PhD, for kindly providing the HCEC line. I would also like to like to thank Dr. Iqbal for mentoring me during this project and Dr. Campbell for kindly providing access to his microscope and workspace.

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