Prevention of Corneal Limbal Epithelial Cell Death during Collagen Crosslinking and the Pathogenic Mechanisms Involved in Progression of Keratoconus.

SYNOPSIS OF THE THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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2010PHXF444P

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PILANI (RAJASTHAN) INDIA

2015

1. Introduction:

Keratoconus (KC) is a progressive degenerative disorder of the cornea resulting in corneal thinning. Worsened stromal thinning and weak corneal stroma results in altered corneal curvature leading to a cone shaped cornea. keratoconus starts at adolescence and progresses in varying manner. Keratoconus is found to be prevalent worldwide, affecting both males and females in all ethnic groups. Keratoconus is always bilateral affecting both the eyes and rarely unilateral. The prevalence reports show that 50 – 230 per 100,000 in the general population are affected with keratoconus. Progression of keratoconus results in loss of visual acuity which cannot be corrected like other refractive errors (eg: myopia can be corrected by glasses). Patients present with keratoconus during teenage years with complaint of sudden or gradual visual blur, astigmatism, monocular diplopia and photophobia.

Over the past decade, corneal collagen cross-linking (CXL) has gained widespread popularity as a technique to confer biomechanical stability to the cornea in eyes with progressive ecstatic disorders, such as keratoconus. Collagen crosslinking is the only procedure which targets the pathophysiology of the disorder. Laboratory research has not only proved the biomechanical and biochemical benefits of corneal CXL, but it has also defined the boundaries of safety when it is applied to the human cornea in the clinical setting. Ocular tissues most vulnerable to damage from UV radiation include the cornea, lens, and retina. The cytotoxicity of the riboflavin–UV-A treatment on keratocytes and endothelial cells has been studied. In the CXL procedure, the epithelium overlying the corneoscleral limbus remains intact. Although, it is the central 7 to 9 mm of the cornea that is directly exposed to UV radiation, it is quite possible that the limbal epithelium also gets exposed to the UV-A radiation. Eye movements during the 30-minute UV-A exposure, deliberate decentration of the CXL procedure to target the peripheral cornea, lateral diffusion of superoxide free radicals from the exposed cornea, and the lack of a compact collimated UV-A beam are all possible situations that could render the limbal epithelium vulnerable to the deleterious effects of exposure. The study was designed to determine the effect of riboflavin–UV-A treatment on the limbal epithelial cells in human cadaveric eyeballs that were subjected to the CXL procedure mimicking the clinical procedure.

The etiology of keratoconus is heterogeneous. Various mode of pathogenesis of keratoconus have been studied namely increased levels of enzyme activities, decreased levels of enzyme

inhibitor, reactive oxygen species, genetic predisposition, magnesium deficiency, keratocyte apoptosis, eye rubbing and use of poorly fitted contact lenses.

Pathogenicity of keratoconus is of unknown etiology hence, is widely studied and has gained importance among ocular researchers for the reason that the progression of the disease is uncontrollable if left untreated.

Chronic keratocyte apoptosis is associated with pathogenesis of keratoconus. Alteration of keratocyte morphology and reduction of keratocyte density is evident in keratoconus cornea. Keratoconus mainly involves the uncontrolled degradation of the corneal extracellular matrix which is enhanced by the proteolytic and collagenolytic enzymes. The loss of keratocytes may be due to the degradation of the extracellular matrix (ECM) or by anoikis mediated cell death in which cells lose their anchorage due to ECM degradation. The effect of degradation and the mediators of ECM degradation is being studied in detail but the effect of the degradation products have not been studied. The homogeneity of the stromal collagen type varies for each corneal layer and 75% of the cornea is composed of type 1 collagen. Type 1 collagen is composed of two α1 chains and one α2 chain, forming a continuous triple helical structure with non- helical telopeptides at both the N-terminal and the C-terminal. C-telopeptides corresponds to the C-terminus region of the alpha-1 chain of type 1 collagen. Collagenolytic enzymes act on the native collagen to release the terminal telopeptides. The terminal telopeptides are involved in the formation of covalent crosslinks between the monomeric collagen molecules. The removal of telopeptides during collagen degradation reduces the strength of the collagen. MMPs and cathepsin K are capable of degrading the triple helix of the collagen but cathepsins L and B are capable of cleaving the telopeptide region of the collagen. Presence of C-telopeptide indicates the collagen degradation and it is also used as biomarker in heart diseases. Only few studies are available discussing the role of collagen degradation products *in vivo.* C- terminal telopeptides in tear of keratoconus patients are considered as the biomarker for the follow up of keratoconus patients. The pathogenic role of these telopeptide on the stromal keratocytes is still unknown.

Extensive clinical studies have been undertaken on keratoconus and have shown that contact lens wearing, eye rubbing, proteolytic enzymes and cytokine production are the main cause of keratoconus. These mechanisms are proposed to injure the corneal epithelial cells leading to apoptosis. Previous litreatures only state the relation between apoptosis and corneal thinning in keratoconus and not on the mediators of apoptosis. The present study was proposed to elucidate the expression of Tumor necrosis factor alpha related apoptosis inducing ligand (TRAIL) in keratoconus corneal epithelial cells and its role in the progressive pathogenesis of keratoconus through TRAIL mediated apoptosis. The apoptotic pathway is composed of extrinsic and intrinsic pathway. The extrinsic pathway is through the death receptors. Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) acts as mediator of apoptosis of cancer cells. TRAIL is a type –II transmembrane protein released by the immune cells. TRAIL mediates apoptosis through the Death receptors DR4, DR5. The death receptors are not expressed in the normal cells and hence the cells survive apoptosis mediated by TRAIL. TRAIL mediates apoptosis by binding to the DR4, DR5 resulting in oligomerisation and activation of Fas ligand which recruits FADD. The caspase cascade is triggered and thereby leading to apoptosis of the cells. TRAIL receptors are only studied to be expressed by cancer cells. Only the cancer cells express TRAIL receptor. The normal cells express only the decoy receptors which compete with death receptors and thereby lead to cell survival. It is a known fact that keratoconus is initially an anterior corneal degenerative disorder characterized with disarranged epithelium and anterior stromal degeneration. The increased expression of TRAIL gene in the keratoconus condition may be the major cause of epithelial damage leading to the death of anterior stomal keratocytes. Thus the present study aims to link TRAIL expression with keratoconus.

2. Scope of the study:

Studies showing the effect of UVA/Riboflavin on keratocytes has been reported in detail but the effect of the same on limbal stem cells are not studied. The study results will help in improving the preventive measure that needs to be taken while performing the procedure in case if the procedure results in limbal stem cell death. PMMA is a material which is widely used to manufacture intraocular lenses. If PMMA ring or the metal can prevent the UVA damage of limbal stem cell then this can result in better management of keratoconus. Till

date studies stress only on the over expression of cytokines, MMPs, cathepsins and the degradation of the stromal collagen by these proteolytic enzymes in the pathogenesis of keratoconus. The effect of the collagen degradation products on the stromal cells are not yet explored. Though the TRAIL death receptors are associated with apoptosis, their distributions in the normal cornea are not reported. The expression levels of TRAIL in normal cornea or the keratoconus cornea and their link with the progression of keratoconus have not been studied yet. Exploring pathogenic mechanisms in keratoconus will lead to newer therapeutic approaches towards keratoconus.

3. Objectives:

The main objectives of the research work are:

1. Determining the effect of Collagen crosslinking on the corneal limbal stem cells in donor cornea before and after exposure to UVA radiation and on covering with PMMA and metal ring.
2. Elucidating the apoptotic effect of the telopeptides on corneal stromal cells *in vitro.*
3. Determining the expression of TRAIL in Keratoconus Corneal Epithelium and to correlate with the progression of keratoconus.

4. Methodology:

The studies undertaken were approved by the Institutes Review Board in accordance with the declaration of Helsinki. Informed consent was obtained from each person before performing the related procedures.

Objective 1:

The cultivability of donor corneal limbal stem cells before and after the CXL procedure was evaluated by cultivating the stem cells on the human amniotic membrane. The effect of CXL on the expression of presumed corneal Limbal stem cell markers were detected by Reverse transcriptase-Polymerase chain reaction on the limbal biopsies. The percentage of viable corneal limbal cells was detected by trypan blue exclusion method.

Objective 2:

The concentration of telopeptides in tears of keratoconus patients was performed using crosslaps ELISA. The comparative analysis and correlation of telopeptide concentration with clinical condition of the patients. Primary cultures of corneal stromal cells were derived from donor cornea and was treated with the synthetic telopeptides. The percentage cell viability was determined by MTT assay and the apoptotic effect was determined by calorimetric TUNEL assay.

Objective 3:

The expression levels of TRAIL in Keratoconus corneal epithelial cells and control corneal epithelial cells were assessed by Reverse transcriptase PCR and Real time PCR. The expression levels of TRAIL in keratoconus corneal epithelial cells were correlated with the keratometry reading and the corneal thickness of keratoconus patients. The Death receptors (DR4, DR5) were demonstrated by immunofluorescence staining.

5. Results and Discussions:

The management of keratoconus has gained importance as the disorder commonly affects the productive age groups (19- 25 Years) of a population. Collagen cross linking treatment is considered as a ray of hope in the management of keratoconus. Series of studies state the safety of collagen cross linking procedure on the corneal layers. The present study stresses on the effect of collagen cross linking (UVA/ Riboflavin) procedure on the corneal limbal epithelial cells. Many different materials are known to prevent the penetration of UV rays, yet according to the feasibility, availability and advantage an appropriate material should be selected. Thus in this study we choose PMMA which was hemi-annular with a thickness of 0.5cm. PMMA was chosen because it is low weight, being easy to handle, rust free and not brittle.

Ten freshly enucleated human cadaveric eyeballs were subjected to the corneal CXL procedure. The cadaveric eye ball was divided into 2 sectors: A and B. Sector A was left unprotected, while sector B was covered by a PMMA shield. Limbal biopsies from both sectors before and after the procedure were analyzed. Each limbal tissue was placed on human amniotic membrane (HAM) to check the cultivability and was subjected to marker

studies using reverse transcriptase polymerase chain reaction. Biopsies collected from both sectors before CXL showed outgrowth of cells on human amniotic membrane. Biopsies collected after exposure from sector A showed no growth on HAM while 2 out of the 10 from sector B covered with the PMMA ring did show outgrowth of cells on HAM. The putative stem-cell marker ATP-binding cassette sub-family G member 2 (ABCG2) was negative in all the samples from sector A after CXL and was positive in 2 out of the 10 samples from sector B. The study showed that collagen crosslinking treatment can result in damage to limbal epithelial cells, particularly the stem cells. Covering the limbal region with PMMA ring offered only partial protection to the limbus against the UV rays during the CXL procedure. Experiments were repeated with metal ring in the place of PMMA ring to compare and to determine their protective effect on the corneal limbal stem cells during CXL procedure. Thirty freshly enucleated human cadaveric eyeballs were subjected to a CXL procedure, mimicking the clinical protocol. Limbal biopsies from sectors A and sector B before and after the procedure were analyzed. Each strip of tissue was divided into 3 segments, for cell count of viable cells, for cultivation on human amniotic membrane, for stem cell and differentiated corneal epithelial cell marker studies using reverse transcriptase–polymerase chain reaction. There was a statistically significant drop in the mean number of viable cells after CXL in sector A but not in sector B. Biopsies from both sectors before CXL and from sector B after CXL showed good growth on human amniotic membrane. Biopsies from sector A after CXL showed no growth on human amniotic membrane. The putative stem cell marker ABCG2 was absent in all samples and p63 was absent in 3 of 10 samples taken from sector A after CXL. The result of our studies showed that cells at all levels of differentiation could be identified in the limbal biopsies taken before the riboflavin-UVA exposure – post mitotic and terminally differentiated cells (TDC) (CK_3/K_{12}- positive), transient amplifying cells (TAC) (Cnx_{43} and Vimentin positive, Involucrine and Integrin 9 positive) and stem cells (p63 and $ABCG_2$ positive). However, after exposure to riboflavin UVA, the $ABCG_2$ expression was negative in 16 biopsies taken from sector A but 19 remained positive in the biopsies taken from Sector B – suggesting a depletion of stem cells in the area which was unprotected by the metal shield. The p63 expression was positive in 8 of 20 biopsies from sector A and in all 15 biopsies from Sector B (Table 4.4). The TAC and TDC cell were less affected, in sector A, where the limbus was unprotected. However, in

sector B, where the limbus was protected by the metal shield, all the epithelial cells markers remained positive. The present study showed that the metal ring conferred complete protection to the limbal region.

Apoptosis of stromal cells are considered to play a main role in the progression of keratoconus. A few studies are available on the mediators of apoptosis in keratoconus. Insight into the mediators of apoptosis will result in the development of therapeutic targets that can control the progression of keratoconus. The present study shows two novel mediators of apoptosis involved in the pathogenisis of keratoconus i.e. C- terminal telopeptides and Tumor necrosis factor alpha related apoptosis inducing ligand (TRAIL). Uncontrolled collagen degradation is the characteristics of keratoconus. Degradation of type 1 collagen releases telopeptides. The physiological concentration was obtained by performing ELISA on the tear samples of keratoconus patients. Human primary corneal stromal cells were cultivated and treated with varying concentrations of synthetic telopeptides (3.012µg, 6.125µg, 12.25µg, 12.25µg, 23.5µg, 47µg and 94µg) and incubated for 24 hours, 48 hours and 72 hours. MTT and TUNEL assays were performed following incubation. The difference between the number of viable cells present in the treated and untreated cells were considered for the analysis. Primary corneal stromal cells treated with varying concentrations of synthetic telopeptide at 24 hours and 48 hours had no morphological or apoptotic changes, the viability remained 100%. The percentage viability was altered after 72 hours of incubation with the synthetic telopeptide. Higher concentration (47µg/ml, 94µg/ml) of telopeptide showed considerable decrease in the cell viability ($p<0.05$, *t test*). The results of the study revealed that the synthetic telopeptide does have an apoptotic effect on stromal cells. Similar phenomenon can be observed in keratoconus stromal cells leading to the progression of keratoconus.

To correlate TRAIL mediated apoptosis with the pathogenesis of keratoconus, forty samples of corneal epithelial cells were collected among which 20 were from keratoconus patient and 20 from myopic patients undergoing Epi LASIK treatment (control group). Reverse transcriptase PCR and Real time PCR was performed to evaluate the expression of TRAIL. The presence of death receptors DR4 and DR5 was demonstrated by indirect immunofluorescence technique on cytospinned corneal epithelial cells and cryostat sections of corneal button from keratoconus patient and a donor. Real time PCR for caspase-8

expression was performed to prove the activated caspase cascade. Statistical analysis of the Real time data was performed using ANOVA. Based on the keratometry readings and corneal thickness, keratoconus eyes were classified as mild, moderate, advanced stages. Reverse transcriptase PCR showed the expression of TRAIL gene in all the 20 keratoconus epithelial cells whereas the control group did not show the expression of TRAIL gene. Fold change of TRAIL expression was higher in case of patients classified under advanced category ($p<0.03$). Keratoconus corneal epithelial cells and the keratocytes showed the expression of DR4, DR5 receptors and TRAIL. But the donor cornea was positive only for the DR4 and negative for DR5 receptor. As the disease progressed the caspase expression was also increased. The level of TRAIL gene expression was found to be increasing as the keratoconus disorder progressed. The results of the present study suggest that the apoptosis of corneal epithelial cells is aided by the expression of TRAIL and TRAIL death receptors DR4, DR5. The analysis of the fold change explained the relation between the expression of TRAIL and the progression of keratoconus. In most of the cases the onset was sudden and the progression was worsening. This further supports the finding that TRAIL gene expression is leading to apoptosis of the corneal cells leading to corneal thinning. Hence it is proved that TRAIL can effectively mediate apoptosis in keratoconus corneal epithelial cells through the death receptors and lead to the pathogenic progression of keratoconus.

6. Specific Contributions of the Study

Specific contributions of the study are:

- The present study has initiated a spark that the limbal stem cells are affected during the collagen crosslinking procedure. Previous literatures had only focused on the safety of corneal keratocytes and endothelium.
- A simple modification in the collagen cross linking procedure i.e. covering the limbal area with metal ring can effectively protect the limbal stem cells from UVA damage.
- The metal ring is now being routinely used in Sankara Nethralaya during the collagen crosslinking procedure for the keratoconus patients.

- Collagen degradation products are considered as diagnostic tool for the detection of keratoconus. But the apoptotic role of telopepteides has not been assessed. The study has assessed the effect of the C- terminal telopepide on the stromal cells, highlighting their apoptotic role.

- TRAIL is widely studied in cancer treatment; this is the first study to correlate the expression of TRAIL with the progression of keratoconus. The present study has shown the distribution of the death receptors in the normal cornea which will aid as reference for future studies. Novel therapeutic ideas can be initiated from the present study on the expression of TRAIL in keratoconus corneas.