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The Expression of Human Corneal MMP-2, MMP-9, proMMP-13 and TIMP-1 in Bullous Keratopathy and Keratoconus

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A B S T R A C T

We aim to find a link between keratokonus (KC) and bullous keratopathy (BK), and extra cellular matrix re-modellation molecules. The activities of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), pro-matrix metalloproteinase-13 (proMMP-13) and tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) were measured using immunoassay in three human corneal tissue layers (epithelium, stroma and endothelium) supernatants of the patients with KC and BK which underwent the perforative keratoplasty. MPP-2, MMP-9, proMMP-13 and TIMP-1 activity was detected in all samples. The epithelial layer showed significantly higher levels of MMP-9 and proMMP-13 in BK than in KC. Increased levels of MMP-2 ($p=0.07$) levels were found in bullous keratopathy compared to keratoconus patients. Epithelial TIMP-1 showed no significant difference in activity between KC and BK. All these findings suggest an active degradation of the extra-cellular matrix in epithelial corneal layer in Bullous Keratopathy. No difference in the concentration of MMP-2, MMP-9, proMMP-13 and TIMP-1 between KC and BK in corneal stroma and endothelium suggest that neither of these molecules play important role in KC or BK pathogenesis, at least not in stroma and endothelium.

Key words: *corneal transplantation, keratoconus, corneal edema, metalloproteinases, tissue inhibitor of metalloproteinase-1*

Introduction

Keratoconus (KC) and bullous keratopathy (BK) are one of the leading indications for corneal transplantation in the past few years[1–5]. Keratoplasty (or corneal tansplantation; grafting) is a procedure in which an abnormal corneal tissue is replaced by a healthy donor cornea. There are full-thickness (penetrating) and partial-thickness (lamellar or deep lamellar) grafting techniques. Keratoplasty can be performed for optical, tectonical, therapeutic and cosmetic reasons. Corneal transplantation is a very successful procedure but not without complications. It seems that early detection and effective prevention of KC and BK could be the best solution but since all the details in the pathogenesis of both BK and KC are not known these patients still undergo corneal transplantation. Tissue-remodeling-involved enzymes seem to have an important role in the pathogenesis of KC and BK[6], thus measuring their activity levels in the corneal tissue could help us to better understand these diseases and it may finally enable us to develop more efficient treatment options to postpone or avoid corneal transplantation. In this study we measured the activity levels of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), pro-matrix metalloproteinase-13 (proMMP-13) and tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) 1 in different corenal layers (epithelium, stroma, endothelium) and evaluated the obtained differences between BK and KC affected eyes.

Bullous keratopathy (BK)

BK is characterized by chronic corneal stromal edema, with or without sub epithelial *bullae* resulting from

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corneal endothelial disease. Most frequently it is due to Fuchs' corneal endothelial dystrophy or corneal endothelial trauma but it is can occur also iatrogenic, usually caused by cataract extraction with intraocular lens implantation. As a result of the stromal and epithelial edema, Bowman's layer and the epithelial basement membrane attenuate or rupture, leading to poor epithelial adhesion and formation of sub epithelial *bullae* that continuously rupture, resulting in recurrent erosions and persistent epithelial defects, exposing the cornea at times to infectious ulcers. A vicious cycle of wound formation and wound healing results in a state of chronic inflammation. In severe cases corneal transplantation is needed, but usually it is not as successful as in KC.

Keratoconus (KC)

Keratoconus is a progressive disorder characterized by corneal thinning and assuming irregular conical shape which induces serious distortion of visual images. Histological analysis displays typical Bowman layer breaks and sub epithelial fibrosis with deposition of various extra cellular matrix (ECM) components. At least 1 of 2000 persons is affected[7,8]. Keratoconus affects young individuals in their late teens or early twenties with variable progression thereafter. The disease usually slowly progresses for 10 to 20 years as the cornea steepens and scars[9]. Although both eyes may be affected, one eye is usually worse than the other. There is no universal theory on pathogenesis of keratoconus. Eran P. et al. found a genetic disorder with variable expression among the carriers in Ashkenazi Jewish family[10]. Low or negligible levels of alpha-enolase in the epithelial cells of keratoconus corneas compared to normal corneas were found and attributed to relatively greater degradation of the two proteins in keratoconus corneas compared to normal corneas in two different studies[11,12]. Decreased levels of lysyl oxidase, enzyme responsible for collagen cross linking, was reported in keratoconus corneas[13]. Keratoconus progression in hypothyroxinaemia during pregnancy was also reported indicating hormonal involvement[14]. We do not know any successful treatment to prevent progression of keratoconus. Visual disorders in mild cases of keratoconus can be successfully treated with glasses or specially designed contact lenses[15]. When vision is no longer satisfactory with glasses or contact lenses, a corneal transplantation may be recommended.

Matrix metalloproteinases (MMP)

The extra cellular matrix (ECM) which represents the majority of corneal tissue not only gives stability and maintains the shape of a cornea but also has a great responsibility for maintaining visual transparency of the corneal tissue. Group of enzymes having important role in the very complex process of ECM degradation are a super-family of Zink-dependent enzymes called matrix metalloproteinases (MMP). Nowadays more than 20 MMP types are described in humans and on the basis of substrate specificity, sequence similarity, and domain organization; they are divided into six groups: collagenases, stromelisins, gelatinases, matrilysins, membrane type MMP (MT-MMP) and other MMP types[16]. Different cell types are involved in producing MMP, but predominantly connective tissue cells and immunological cells. Under normal physiological conditions, as far as we know, the activities of MMPs are precisely regulated at the level of transcription, activation of the precursor zymogens, interaction with specific ECM components and inhibition by endogenous inhibitors[16–18]. MMPs can be activated by proteinases or in vitro by chemical agents.

Matrix metalloproteinase-13 (MMP-13), also known as collagenase III, is secreted collagenase that plays a physiological role in the degradation of extra cellular matrix found in skeletal tissues. Ye and al.[19] suggested that MMP-13 may play an important role in the MMP-9-associated proteolytic cascade that allows rapid turnover of the ECM components during corneal wound healing.

Matrix metalloproteinase-2 (MMP-2), also known as Gelatinase A, is secreted as proMMP-2 which is not readily activated by general proteinases. The main activation of proMMP-2 takes place on the cell surface and is mediated by MT-MMPs. MMP-2 is also activated by MMP-1 and MMP-13[16]. Gelatinases (MMP-2 and MMP-9) degrade denatured collagen, gelatin, native type IV, V and VII collagens as well as other ECM components[16,20–22]. MMP-2 also digests fibrillar type I and II collagens[23].

Matrix metalloproteinase-9 (MMP-9), also known as Gelatinase B, is secreted as proMMP-9, activated by plasmin, MMP-3, MMP-13 and MMP-26. Transforming growth factor β (TGFβ), tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), lipopolysaccharide (LPS) and endothelial growth factor (EGF) are known as inducers of the MMP-9. Higher levels of MMP-9 have been found in the corneal epithelium of the patients with pseudophakic corneal edema[24].

Tissue inhibitors of matrix metalloproteinases (TIMP)

Tissue Inhibitors of MMPs (TIMP) are multifunctional mollecules involved in specific reversible inhibition of MMP activity but also have other roles in cell cyclus[18,25]. They bind MMPs in a 1:1 stoichiometry so changes of TIMP levels directly affect the level of MMP activity. Four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified in vertebrates[26]. Their expressions vary during development and tissue remodeling.

Materials and Methods

Corneal tissue buttons collected after penetrating keratoplasty of fourteen consecutive patients (eyes) with bullous keratopathy (7 patients) and keratoconus (7 patients) were micro surgically divided into three layers: epithelium, stroma and endothelium with Descemet membrane. Pachymetry and diameter of each tissue sample were measured and tissue volume calculated. Tissue samples were separately incubated in 0.5 milliliters of tissue culture (Cornea Prep® solution, Eurobio, France)

for 24 hours at 37 °C and after stored at –80 °C for further examination.

Immediately after defrosting, the levels of activity of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), pro- matrix metalloproteinase-13 (pro--MMP-13) and tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) were measured in tissue sample supernatants by enzyme linked immunosorbent assay (ELISA, Quantikine®, R&D Systems Inc).

The data were analyzed using statistical package »Statgrasphics plus 4.0 for Windows«. The data was presented as mean ± sem (standard error of mean). The significance difference of compared samples was determined using student t-test.

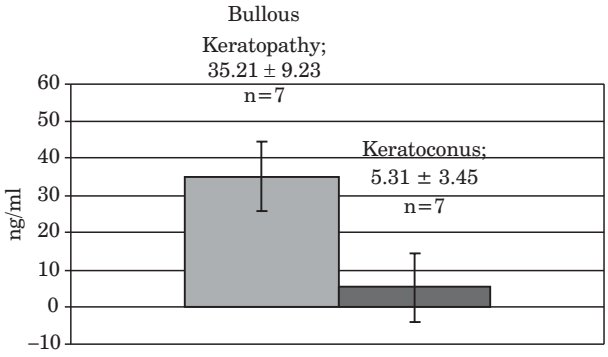

Fig. 1. Average concentrations of MMP-9 in corneal epithelial tissue supernatants in Bullous Keratopathy and Keratoconus (p=0.010).

Results

MMP-2, MMP-9, pro MMP-13 and TIMP-1 activity in all three corneal layers of the corneal tissue supernatant samples both for bullous keratopathy (Table 1) and keratoconus (Table 2) were measured.

Epithelial tissue supernatants showed significantly higher concentration of MMP-9 in BK (35.12±9.23 ng/mL) than in KC (5.31±3.45 ng/mL, n=7) (p =0.010, Figure 1). Epithelial tissue supernatants also showed significantly higher concentration of proMMP-13 in BK (301.73±75.81 pg/mL, n=6) than in KC (90.69±30.61 pg/mL, n=6) (p = 0.027, Figure 2).

MMP-2 concentration in epithelial layer was much higher (7.02±3.08, n=6) in BK patients than in KC patients (1.44±0.50 (n=6) (p=0.078, Figure 3). TIMP-1 concentration in epithelial tissue supernatants showed increased, but not significant, levels in BK as compared to KC (Tables 1, 2).

Stromal and endothelial layer tissue supernatants showed no significant difference in MMP-2, MMP-9, proMMP-13 and TIMP-1 concentration between BK and KC (Tables 1, 2).

Discussion

Several surgical techniques of corneal transplantation are nowadays reasonable, but far from ideal, therapy for treating patients with keratoconus and bullous keratopathy. Better understanding of the pathogenesis of these diseases would allow us to predict and on time effectively prevent or even cure affected patients. MMPs may be one of the factors involved in the pathogenesis of mentioned diseases. In this study we have found that the corneal epithelial concentrations of MMP-9 and proMMP--13 are significantly higher in BK than in KC; that MMP-2 epithelial concentration is also higher in BK than

TABLE 1
AVERAGE MMP-2, MMP-9, PROMMP-13 AND TIMP-1
CONCENTRATION IN HUMAN CORNEAL EPITHELIUM, STROMA AND ENDOTHELIUM LAYERS IN BULLOUS KERATOPATHY

	MMP-2 (ng/mL)	MMP-9 (ng/mL)	proMMP-13 (pg/mL)	TIMP-1 (ng/mL)
Epithelium	7.02 ± 3.08 (n=6)	35.21 ± 9.23 (n=7)	301.73 ± 75.81 (n=6)	0.60 ± 0.24 (n=7)
Stroma	55.72 ± 13.50 (n=7)	1.82 ± 0.47 (n=7)	406.78 ± 244.10 (n=7)	15.06 ± 2.78 (n=7)
Endothelium	4.27 ± 2.27 (n=7)	1.07 ± 0.22 (n=7)	195.79 ± 105.58 (n=7)	0.80 ± 0.25 (n=7)

MMP-2 – matrix metalloproteinase-2, MMP-9 – Matrixmetalloproteinase-9, proMMP-13 – pro matrix metalloproteinase-13, TIMP-1 – tissue inhibitor of matrix metalloproteinases-1

TABLE 2
AVERAGE MMP-2, MMP-9, PROMMP-13 AND TIMP-1 CONCENTRATION IN HUMAN CORNEAL EPITHELIUM,
STROMA AND ENDOTHELIUM LAYERS IN KERATOCONUS

	MMP-2 (ng/mL)	MMP-9 (ng/mL)	proMMP-13 (pg/mL)	TIMP-1 (ng/mL)
Epithelium	1.44 ± 0.50 (n=6)	5.31 ± 3.45 (n=7)	90.69 ± 30.61 (n=6)	1.28 ± 1.00 (n=7)
Stroma	63.26 ± 3.66 (n=7)	2.02 ± 0.88 (n=7)	156.34 ± 42.85 (n=7)	15.58 ± 3.04 (n=7)
Endothelium	12.99 ± 3.43 (n=7)	1.00 ± 0.14 (n=7)	81.53 ± 23.79 (n=7)	2.67 ± 1.50 (n=7)

MMP-2 – matrix metalloproteinase-2, MMP-9 – Matrixmetalloproteinase-9, proMMP-13 – pro matrix metalloproteinase-13, TIMP-1 – tissue inhibitor of matrix metalloproteinases-1

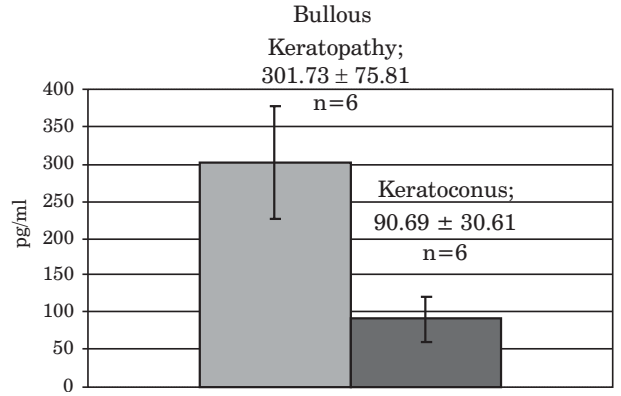

Fig. 2. Average concentrations of proMMP-13 in corneal epithelial tissue supernatants in Bullous Keratopathy and Keratoconus (p=0.027).

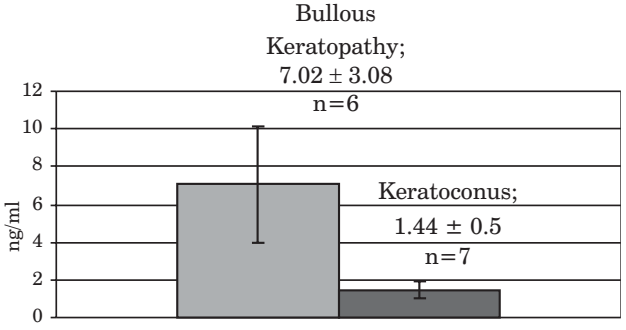

Fig. 3. Average concentrations of MMP-2 in corneal epithelial tissue supernatants in Bullous Keratopathy and Keratoconus (p=0.078).

in KC (yet not significantly, p=0.07) and that there was no difference in TIMP-1 epithelial concentration between KC and BK.

MMP-9, the level of which we have proved to be increased in BK, cleaves mostly collagen types IV and V[23,27]. This finding could partially explain bullous changes and recurrent erosions in BK, since collagen IV which forms epithelial basal lamina, is cleaved by MMP-9. The higher concentration of MMP-9 in bullous keratopathy is contributed by increased levels of NO in edematous (thus hypoxic) corneal tissue[28] and increased levels of pro-inflammatory cytokines like TGFβ, TNF-α, IL-1β, LPS and EGF as known inducers of the MMP-9. Increased MMP-9 levels, with hypoxia could be also responsible for increasing VEGF levels and new blood vessels ingrowth[29] in BK. Our results further suggest that we could slow down or even stop formation of *bullae,* erosions and new vessels in BK and postpone keratoplasty, by stopping the activity of MMP-9.

In corneal stroma and endothelium we haven't found any significant difference in expression of MMP-2, MMP-9, proMMP-13 and TIMP-1 between BK and KC. When compared to findings in epithelium, this finding implies that in BK extracellular matrix degradation is the most intense in corneal epithelial layer and that MMP-2, MMP--9, proMMP-13 and TIMP-1 play a minor role in the pathogenesis of both diseases, at least in stromal and endothelial layer.

Mackiewicz et al. found increased levels of MMP-13 in keratoconus corneas[6] and our findings suggest not normal (at least not increased) concentration of proMMP-13 in KC which implies that MMP-13 proteinase inhibitor/activator cascade may be affected in keratoconus.

Conclusion

Higher MMP-2 and significantly higher activity levels of MMP-9 and proMMP-13 were found in human corneal epithelial layers in patients with bullous keratopathy than in keratoconus patients. These findings indicate that there is an active degradation of the extracellular matrix in epithelial corneal layer in bullous keratopathy. No MMP-2, MMP-9, proMMP-13 and TIMP-1 difference in concentration between KC and BK in corneal stroma and endothelium suggest that neither of these molecules play important role in KC or BK pathogenesis, at least not in stroma and endothelium.

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EKSPRESIJA MMP-2, MMP-9, PROMMP-13 I TIMP-1 KOD BULOZNE KERATOPATIJE I KERATOKONUSA

S A Ž E T A K

Keratokonus (KC) i Bulozna keratopatija (BK) uzrokuju značajne vidne smetnje i među najčešćim su indikacijama za transplantaciju rožnice u Hrvatskoj i u svijetu u zadnjih nekoliko godina. Naš je cilj u ovom radu pronaći vezu između ovih poremećaja i enzima koji sudjeluju u pregradnji izvanstanične tvari, matrix metalloproteinaza (MMP). Aktivnost matrix metaloproteinaze-2 (MMP-2), matrix metaloproteinaze-9 (MMP-9), pro-matrix metaloproteinaze-13 (proMMP-13) i tkivnog inhibitora matrix metaloproteinaza-1 (TIMP-1) mjerene su metodom enzyme linked immunosorbent assay (ELISA) u supernatantima tri sloja rožnice (epitel, stroma, endotel) pacijenata oboljelih od KC i BK koji su prethodno podvrgnuti perforativnoj keratoplastici. Aktivnost MPP-2, MMP-9, proMMP-13 i TIMP-1 je detektirana u svim promatranim uzorcima. Za razliku od TIMP-1 gdje nije uočena razlika, koncentracija MMP-9 i proMMP-13 u rožničnom epitelu je bila statistički značajno viša u pacijenata oboljelih od BK, nego u onih oboljelih od KC. Povišene koncentracije MMP-2 ($p=0,07$) također su zabilježene u rožničnom epitelu pacijenata oboljelih od BK, nego u onih oboljelih od KC. Sve ovo upućuje da je degradacija epitelnog ekstracelularnog matriksa kod bulozne karetopatije vrlo živ proces. U rožničnoj stromi i endotelu nisu pronađene značajne razlike u koncentraciji MMP-2, MMP-9, TIMP-1 i proMMP-13 između KC i BK što ukazuje da ni jedna od ovih molekula vjerojatno nema značajniju ulogu u patogenezi ovih oboljenja.