UDK 617.713-089.843
Original scientific paper
Received: 18 January 2016
Accepted: 23rd March 2016

GENE EXPRESSION ANALYSIS OF MATRIX METALLOPROTEINASE 2, MATRIX METALLOPROTEINASE 13 AND TISSUE INHIBITOR OF MATRIX METALLOPROTEINASE 1 IN EPITHELIUM OF DISEASED HUMAN CORNEAS*

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Summary

It has been shown in animal models that matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are responsible for remodelling and neovascularization of the diseased cornea. Under normal conditions, MMPs are expressed at relatively low levels, which may increase in case of different corneal diseases. In this paper we have investigated gene expression of matrix metalloproteinase 2, matrix metalloproteinase 13 and their inhibitor TIMP-1, in keratoconus, bullous keratopathy and vascularized corneal scar; aiming to determine whether these molecules are differentially expressed in epithelium of diseased human corneas. Keratoconus, bullous keratopathy and vascularized corneal scar were chosen for two reasons: they represent low-, intermediate- and high-risk corneal disease regarding corneal graft rejection rate (respectively) and they are very common reason for corneal transplantation. Our results have show that in bullous keratopathy, expression of both MMP-13 and MMP-2 genes were significantly increased compared to vascularized corneal scar and keratoconus; however only MMP-13 reached significance in our relatively small human sample. These results are comparable to our previously published data on protein levels of MMPs in diseased human corneal epithelium, where we showed significant increase of MMP-13 and MMP-2 proteins in patients with bullous keratopathy compared to keratoconus. TIMP-1 gene expression remained constant across all groups of patients, impli-

* This research was funded by Croatian Ministry of Science, Education and Sports, Grant No. 0982464-1647, Grant No. 0982464-2460 and Grant No. 0000000-0222.

cating that this molecule does not play significant role in pathological epithelial changes during keratoconus, bullous keratopathy and vascularized corneal scar. Our results may be useful in development of new therapeutic agents both to treat pathological corneal changes and to prevent corneal graft rejection after corneal transplantation.

***Keywords*:** cornea; matrix metalloproteinase; keratoconus; bullous keratopathy; vascularized corneal scar.

INTRODUCTION

Healthy cornea is avascular and transparent in order to provide good vision. It consists mainly of extracellular matrix (ECM) predominantly built of different types of collagen [1]. In case of corneal disease, highly organized corneal architecture is disrupted, the extracellular matrix (ECM) is degraded, and new disorganized ECM is formed, resulting in the loss of corneal transparency [2].Matrix metalloproteinases (MMPs) are extracellular endopeptidases which have a key role in ECM remodelling; they are controlling intercellular interactions and interactions of cells with ECM [3]. More than 20 members of human MMP family has been identified and classified into five subgroups: gelatinases, collagenases, matrilysins, stromelysins and membrane-type MMPs. Under normal conditions, MMPs are expressed at relatively low levels, which may increase in case of different corneal diseases. MMP-2, MMP-9, MMP-13 and MMP-14, for example, have been shown to participate in corneal wound healing and in the pathogenesis of inflammatory corneal diseases [4-8]. Tissue inhibitors of matrix metalloproteinase (TIMPs) are specific inhibitors of MMPs which regulate and control the activity of MMPs in tissues [9-12].

In the advanced stage of many corneal diseases, corneal transplantation (CT) is needed to restore normal corneal transparency. Based on the inflammatory profile of their corneas at the time of CT patients are usually divided to be of low-risk (keratoconus - KC), intermediate risk (bullous keratopathy - BK) or high-risk (vascularized corneal scar - VCS) for postoperative graft rejection. Keratoconus is a non-inflammatory corneal thinning followed by anterior protrusion of the cornea, for which aetiology is not known and various genetic and environmental factors have been suggested [13,14]. Bullous keratopathy is a corneal disorder characterized by corneal stromal oedema with epithelial bullae formation due to endothelial cell loss decompensation; while vascularised corneal scar represents typical inflammatory corneal condition in which prolonged inflammation and neovascularisation lead to the loss of corneal transparency [15].

To aid in developing efficacious post-CT therapies for those patients, it is important to understand and further investigate the complex pathways related to MMP and TIMP signalling in particular human corneal disease [16,17]. Therefore, we have

investigated gene expression of MMPs and TIMPs in the corneal epithelium of patients suffering from KC, BK and VCS at the time of CT. Although it has been shown in animal model that those molecules promote pathological cellular interactions leading to remodelling and neovascularization of corneal tissue, it is still not known whether they are differentially expressed in high-, intermediate and low-risk human corneal diseases [18,19].

MATERIALS AND METHODS

Tissue preparation

With the proper informed consent and approval by the Ethical Committee of our Hospital human corneal epithelium was taken *ex vivo* from 18 recipient corneal buttons obtained during corneal transplantation (7 diagnosed with keratoconus, 6 with bullous keratopathy and 5 with vascularized corneal scar). Corneal epithelium was separated from the corneal stroma by fine forceps under the operating microscope, and homogenized using the TissueRuptor homogenizer (Qiagen, Germany). Control healthy epithelium (n=5) was collected from healthy corneas obtained from the eye bank.

RNA isolation

Total human corneal epithelial RNA was extracted from the individual corneal epithelium using Rneasy Mini Kit (Qiagen, Hilden, Germany); cDNA was prepared from 1 μg of purified total RNA in 20 μl reaction volume, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to manufacturer's instructions. Real-time PCR analysis was carried out on an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA) to quantify relative mRNA expression of matrix metallopeptidase 2 (MMP-2), matrix metallopeptidase 13 (MMP-13) and tissue inhibitor of MP-1 (TIMP-1) genes, respectively. Assay IDs used for the analysis are shown in Table 1. The housekeeping gene gapdh was used as an endogenous control to normalize the total amount of cDNA in each sample. Reactions were carried out in a total volume of 20 μl using TaqMan® Gene Expression Master Mix reagent (Applied Biosystems, Foster City, CY, USA) and 5 μl of cDNA for template with forward and reverse primers. Using the $2^{-\Delta\Delta Ct}$ method, data are presented as the fold-change in gene expression normalized to an endogenous reference gene and relative to the control. All reactions were carried out in triplicate [20].

Table 1. Primers used for quantitative real-time PCR analysis

Gene	Assay ID	Product size (bp)
GAPDH	Hs99999905_m1	122
MMP2	Hs01548733_m1	84
MMP13	Hs00233992_m1	91
TIMP1	Hs00171558_m1	104
VEGFA	Hs00900058_m1	81
VEGFR1	Hs01052936_m1	72

Statistical analysis

Statistical analyses of data were performed using R v2.15.3 (CRAN, http://cran.r-project.org) and RStudio for Windows, v0.97 (http://www.rstudio.com/). For the analysis of real-time PCR data, all groups were tested for normality of distribution using Shapiro-Wilk test. The differences between multiple groups were compared with Kruskall-Wallis non-parametric ANOVA, followed by Wilcoxon signed-rank test for testing differences between two related groups. For all tests significance level was set at $p < 0.05$.

RESULTS

MMP-2 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar

The expression of MMP-2 gene remained unchanged in patients with keratoconus, bullous keratopathy and vascularised corneal scar when compared to control patients, as revealed by Kruskal-Wallis non-parametric ANOVA (Kruskal-Wallis chi-squared = 6.8404, df = 3, p-value = 0.07716) (Fig. 1).

MMP-13 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar

The MMP-13 gene expression was not determined in patients with keratoconus because the expression of the MMP-13 gene was below detection limit of the method. Kruskal-Wallis non-parametric ANOVA has shown significant differences in MMP-13 gene expression between BK and VCS groups (Kruskal-Wallis chi-squared = 6, df = 2, p-value = 0.049). In patients with bullous keratopathy MMP-13 gene was significantly upregulated, as revealed by Wilcoxon rank-sum test (W=8, p = 0.012) (Fig. 2).

TIMP-1 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar

TIMP1 gene expression remained unchanged in any of groups, when compared to control samples (Kruskal-Wallis chi-squared = 5.5606, df = 3, p-value = 0.1351) (Fig. 3).

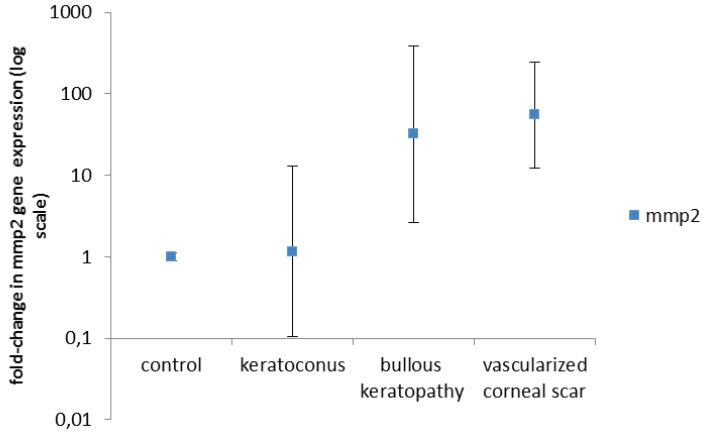

Figure 1. Real-time PCR analysis of MMP-2 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar. Fold-change in gene expression compared to control was calculated using the $2^{-\Delta\Delta CT}$ method as described in Materials and methods. Data are presented as fold-change ±SE on log scale.

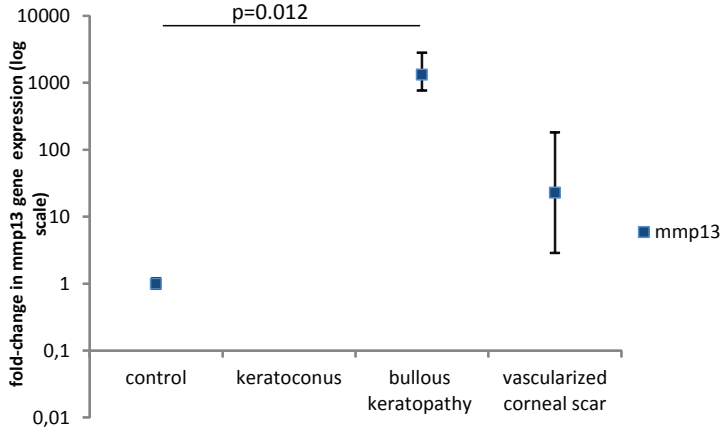

Figure 2. Real-time PCR analysis of MMP-13 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar. Fold-change in gene expression compared to control was calculated using the $2^{-\Delta\Delta CT}$ method as described in Materials and methods. Data are presented as fold-change ±SE on log scale.

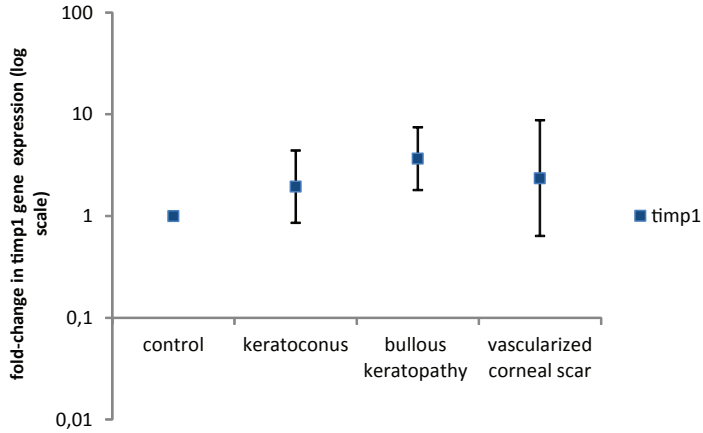

Figure 3. Real-time PCR analysis of TIMP-1 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar. Fold-change in gene expression compared to control was calculated using the $2^{-\Delta\Delta CT}$ method as described in Materials and methods. Data are presented as fold-change ±SE on log scale.

DISCUSSION

Corneal transplantation (CT) is one of the most frequently performed transplantations [21]. However, the molecular mechanisms leading to corneal opacification in different corneal diseases are still not well determined. From the clinical experience we know that the outcome of CT significantly varies according to the characteristics of the recipient cornea where the corneal graft is placed. Therefore, it is of great importance to understand which molecules are involved in pathological changes of corneal tissue in different diseases; both to invent new topical agents to treat those diseases and to improve the surgical prognosis of CT. Based on the experimental data in animal models it is clear that MMPs play an important role in changes of ECM and thus transparency of the cornea, and that their activity is regulated via TIMPs [8-12,22,23]. However, very little data on MMPs and TIMP role in changes of corneal transparency are available for human corneas [24-27]. For this reason we were interested whether any changes in gene expression of MMPs and TIMPs could be detected in human low-risk disease like keratoconus (KC) versus intermediate and high-risk ones like bullous keratopathy (BK) and vascularized corneal scars (VCS). In the study of Gordon et al., an comprehensive analysis for the entire MMP gene expression during mouse corneal abrasion injury was done, and it has been shown that mRNA levels for several MMPs and TIMP-1 were significantly upregulated during epithelial migration, while MMP-13 remained elevated even after

epithelialization suggesting that MMP-13 may be a marker of prolonged corneal epithelial injury [12]. In our study, we have shown that MMP-13 gene is significantly upregulated also in human epithelium of patients with BK, but not in KC and VCS, suggesting specific role of MMP-13 in epithelial corneal changes in patients with BK. In our previously published paper, we have also investigated protein levels of different MMPs and TIMPs in BK and KC corneas, and have similarly shown that epithelium of BK patients show higher level of MMP-13 protein compared to KC [27]. Moreover, we have shown that protein levels of epithelial TIMP-1 remained unchanged in both KC and BK [27]. This finding is also confirmed by gene expression analysis in this study, where no difference was found in TIMP-1 gene expression between BK, KC and VSC corneas. Although MMP-2 gene analysis in this study showed higher gene expression in the epithelium of BK and VCS patients compared to KC, this difference was not statistically significant. However, our previous protein analysis of MMP-2 showed increased MMP-2 levels in patients with BK compared to KC [27]. Current results may have been influenced by the relatively small sample in this study, so further investigations would be beneficial for confident conclusion whether MMP-2 has a role in corneal epithelial changes in BK patients. Overall, one may conclude that MMP-13 plays a role in pathological changes of corneal epithelium in BK, since both MMP-13 gene and protein are highly expressed in corneal epithelium of such patients.

CONCLUSION

Presented results revealed that the expression of MMP-13 gene is significantly increased in bullous keratopathy (BK), somewhat increased in vascularized corneal scar (VCS) and under levels of detection in keratoconus (KC). Although MMP-2 gene expression was increased in patients with BK and VCS compared to keratoconus, this difference did not reach significance in this relatively small sample. TIMP-1 gene expression remained constant across all groups of patients.

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Saţetak

Genska ekspresija matriks metaloproteinaze 2, matriks metaloproteinaze 13 te tkivnog inhibitora matriks metaloproteinaze 1 u epitelu oboljelih ljudskih rožnica

Matriks metaloproteinaze (MMP) i njihovi inhibitori (TIMP) smatraju se čimbenicima odgovornim za remodelaciju i neovaskularizaciju rožnice do koje dolazi kod raznih bolesti, što je pokazano na životinjskim modelima. U zdravim rožnicama matriks metaloproteinaze izražene su u relativno niskim koncentracijama te rastu u slučaju rožničnih bolesti. U ovome smo radu istražili gensku ekspresiju matriks metaloproteinaze 2 i 13 u ljudskim bolestima rožnice keratokonusu, buloznoj keratopatiji i vaskulariziranim ožiljcima rožnice kako bismo stekli uvid u to imaju li ti MMP-ovi značenje u razvitku humanih bolesti rožnice. Keratokonus, bulozna keratopatija i vaskularizirani ožiljci odabrani su iz dva razloga: zbog toga što klinički predstavljaju bolesti niskog, intermedijarnog i visokog rizika glede ishoda transplantacije rožnice te zbog toga što su to učestale indikacije zbog kojih se obavlja transplantacija rožnice. Naši rezultati pokazuju da u slučaju bulozne keratopatije dolazi do povišene ekspresije i MMP-13 i MMP-2 gena u usporedbi s keratokonusom i vaskulariziranim ožiljcima; međutim samo je povišena ekspresija MMP-13 bila signifikantna u našoj relativno maloj skupini bolesnika. Ti rezultati podudaraju se s našim prethodnim istraživanjima u kojima smo proteinskom analizom dokazali da su i MMP-13 i MMP-2 protein signifikantno više producirani u epitelu rožnica bolesnika s buloznom keratopatijom nego u onih s keratokonusom. Razina ekspresije TIMP-1 gena nije se razlikovala među ispitivanim uzorcima ukazujući na to da ta molekula nema bitnu ulogu u patološkim promjenama epitela u slučaju keratokonusa, bulozne keratopatije i vaskulariziranih ožiljaka. Dobiveni rezultati mogli bi biti korisni u razvitku novih terapija kako za liječenje patoloških rožničnih promjena, tako i za liječenje reakcije odbacivanja transplantata rožnice.

Ključne riječi: rožnica; matriks metaloproteinaze; keratokonus; bulozna keratopatija; vaskularizirani ožiljci rožnice.

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