Original Article

Quantitative Analysis of Transforming Growth Factor β Isoforms mRNA in the Human Corneal Epithelium

(TGF-β isoforms / real-time RT-PCR / mRNA – quantification / corneal epithelium)

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Abstract. TGF-β is an important mediator of cell growth, differentiation, and proliferation and plays a significant role in both normal and pathological corneal tissue. However, the quantitative relations between TGF-β1, -β2 and -β3 isoforms in human cornea still remain unclear. Therefore, the aim of this study was to determine the gene expression profile of *TGF-βs* in order to evaluate quantitative relations between the examined transcripts in human corneal epithelium. Transcriptional activity of *TGF-β1*, *2*, *3*, *GAPDH* and *β-actin* genes was estimated on the basis of mRNA copy number per 1 μg of total RNA using the real-time QRT-PCR technique with the SYBR Green I chemistry. Specificity of RT-PCR reaction was confirmed by determination of the characteristic melting temperature for each amplimer. Additionally, the RT-PCR products were separated on 6% polyacrylamide gels and visualized with silver salts. Expression of all *TGF-β* genes for the corneal epithelium was determined. Comparable analysis of mRNA copies/1 μg of total RNA for each TGF-β isoform showed that: TGF-β1 > TGF-β2; TGF-β3 > TGF-β2; TGF-β1 = TGF-β3 (ANOVA test P < 0.0001; post-hoc Tukey's test: TGF-β1 and TGF-β2, P = 0.0306; TGF-β3 and TGF-β2, P = 0.0045; TGF-β1 and TGF-β3 NS). We found different expression of the TGF-β1, -2 and -3 isoforms in the human corneal epithelium. Such differential expression of TGF-βs suggests that each of them may play a specific role in corneal tissue.

Introduction

Cornea is a highly specialized and unique connective tissue that combines transparency, refractive power for correct vision, tensile strength, and protection against infections (Chen et al., 2000; Chakravarti, 2001; 2004). Features of this tissue are maintained by various factors and mechanisms associated with the growth, differentiation, proliferation, and cell death. Such processes depend on the organized activities of a variety of cytokines, including growth factors, interleukins, and extracellular matrix proteins (Chen et al., 2000; Hayashida-Hibino and Watanabe, 2001; Tuli et al., 2006). One of the most important mediators is constituted by the family of transforming growth factor β (TGF-β) composed of five isoforms (TGF-β1–5) (Jakowlew et al. 1988; Ten et al., 1988; Lyon and Moses, 1990). Among them only TGF-β1, -β2, and -β3 are found in humans (Lyon and Moses, 1990; Hayashida-Hibino and Watanabe, 2001). All isoforms are encoded by unique genes of various chromosome locations (Elliot and Blobe, 2005) and revealed 64–85% amino acid sequence homology (Frank et al., 1996). They are present in epithelial, stromal, and endothelial layers of the cornea (Sporn and Roberts, 1992; Pasquale et al., 1993; Nishida et al, 1995; Honma et al., 1997; Hayashida-Hibino and Watanabe, 2001; Carrington et al., 2006). However, the coordinated changes of mRNA levels of the three specific TGF-βs in these multiple structures have not been clarified so far.

The pivotal role played by TGF-β isoforms both in corneal development and in normal and abnormal wound healing, including epithelial migration and scar formation, was emphasized repeatedly (Jester et al., 1999; Saika et al., 2001; Kawakita et al., 2005; Tuli et al., 2006). Moreover, these cytokines stimulate neovascularization, are involved in the formation of extracellular matrix and fibrosis in the eye and many other tissues (Border and Noble, 1994; Chen et al., 2000). They have also been shown to be important in ocular scarring in conditions including proliferative vitreoretinopathy (Connor et al., 1989; Kon et al., 1999), cataract formation (Hales et al., 1995), corneal opacities (Chen et al., 2000), subconjunctival scarring or complication of filtration surgery in glaucoma (Cordeiro, 2002; 2003). For better understanding the aethiopathogenesis of eye diseases it seems to be important to disclose gene expres-

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Abbreviations: GAPDH – glyceraldehyde-3-phosphate dehydrogenase, TGF-β – transforming growth factor β.

sion patterns in anatomical compartments and structures of the human eye.

In the current study we investigated transcriptional activity of TGF-β isoforms in the normal human corneal epithelium. We answered the questions as to which studied genes were subjected to expression and how quantitative relationships between mRNA levels of these three isoforms were observed in this structure.

Material and Methods

Dissection of human corneas

Normal human corneas were obtained from 20 donors (both men and women) whose age ranged from 19 to 63. In each case cornea was taken in the course of the two hours *post mortem*. Inclusion criteria for becoming corneal tissue donors were determined by The Eye Bank Association of America (EBAA).

Corneal rims remained after keratoplasty and the epithelium was stripped from the stroma and stored in EUSOL C (Alchimia, Padova, Italy) at –70°C for 24 h. The research was approved by the local Bioethics Committee.

RNA extraction from tissue specimens

Total RNA was isolated from the corneal epithelium applying a commercially available kit (Total RNA Prep Plus A&A Biotechnology, Gdansk, Poland) according to the manufacturer's instructions based on the modified Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987). Quality of extracts was checked electrophoretically using 0.8% agarose gel stained with ethidium bromide. The results were analysed and registered using gel documentation system 1D Bas-Sys (Biotech-Fisher, Perth, Australia). In case of contamination of the RNA extracts with genomic DNA, they were treated with DNAase I (MBI Fermentas, Vilnius, Lithuania) according to manufacturer's instructions. The RNA concentration was determined by spectrophotometric measurement in 5-µl capillaries using a Gene Quant II RNA/DNA Calculator (Pharmacia Biotech, Cambridge, UK).

Design of specific primers

Oligonucleotide primers specific for TGF-β1 (GenBank accession no. X02812) (Derynck et al., 1985), TGF-β2 (GenBank accession no. NM_003238) (Thompson et al., 2006) and TGF-β3 (GenBank accession no. NM_003239) (Ye et al., 2006) isoforms were designed using Primer Express ™ Version 2.0 software (PE Applied Biosystems, Inc., Foster, CA) (Table 1).

Real-time QRT-PCR assay

Transcriptional activity of *TGF-β1*, *2*, *3*, *GAPDH* and *β-actin* genes was evaluated on the basis of mRNA copy number/1 µg of total RNA by the use of the real-time QRT-PCR technique with the SYBR Green I chemistry (SYBR Green Quantitect RT-PCR Kit, QIAGEN, Valencia, CA). Analysis was carried out using an Opticon™ DNA Engine Sequence Detector (MJ Research Inc., Watertown, MA). QRT-PCR assay was performed in triplicate for each of 20 samples. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were included to monitor RT-PCR efficiencies for all samples. Gene expression of *GAPDH* and *β-actin* were analysed using specific primers (Table 2).

The thermal profile was as follows: 50°C for 30 min for reverse transcription, 95°C for 15 min, 50 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. To simultaneously detect the expression profile of examined genes we used a commercially available fragment of cDNA β-actin (TaqMan® DNA Template Reagents Kit and β-actin Control Reagent Kit, PE Applied Biosystems) in five different concentrations (from 0.2×10^3 to 4.0×10^3 copy per 1 µl) as recommended by Bustin

Table 1. Characteristic of primers used for amplification

Gene name	Sequence of primers (5'-3')	Length of amplicon (bp)	TM (°C)
TGF-β1	Forward: TGAACCGGCCTTTCCTGCTTCTCATG Reverse: GCGGAAGTCAATGTACAGCTGCCGC	151	85
TGF-β2	Forward: TACTACGCCAAGGAGGTTTACAAA Reverse: TTGTTCAGGCACTCTGGCTTT	201	80
TGF-β3	Forward: CTGGATTGTGGTTCCATGCA Reverse: TCCCCGAATGCCTCACAT	121	81

Table 2. Characteristic of primers used as internal controls for amplification

Gene name	Sequence of primers (5'-3')	Length of amplicon (bp)	TM (°C)
GAPDH	Forward: GAAGGTGAAGGTCGGAGTC Reverse: GAAGATGGTGATGGGATTC	226	80
β-actin	Forward: TCACCCACACTGTGCCCATCTACGA Reverse: CAGCGGAACCGCTCATTGCCAATGG	295	85

TM – melting temperature; bp – base pairs

(2002). Amplification plots for each dilution of the control template were used to determine the Ct value. A standard curve was generated by plotting the Ct values against the log of a known amount of β-actin cDNA copy numbers. Each run was completed with a melting curve analysis to confirm the specificity of the amplification and the absence of primer dimers. The RT-PCR products were separated on 6% polyacrylamide gels and visualized with silver salts.

Statistical analysis

Results were subjected to statistical analysis using Statistica PL 5.0 software (StatSoft, Krakow, Poland) with the significance level set at $P < 0.05$. The values were expressed as median. Nonparametric ANOVA Friedman test and post-hoc Tukey's test were applied to compare the differences between the expression level of studied genes. Variations of results in each experiment were assessed by coefficient of variation – Cv, whereas homogeneity of variances was determined using the Levene's test.

Results

In this study we analysed mRNA expression in human corneal epithelium for two housekeeping genes (*GAPDH* and *β-actin*) and three isoforms of the *TGF-β* family by the real-time QRT-PCR. RT-PCR specificity was confirmed experimentally based on melting temperatures of the amplimers (Fig. 1A, 1B) and by polyacrylamide (PAA) electrophoreses (Fig. 2). In all samples tested, mRNA of GAPDH and mRNA of β-actin

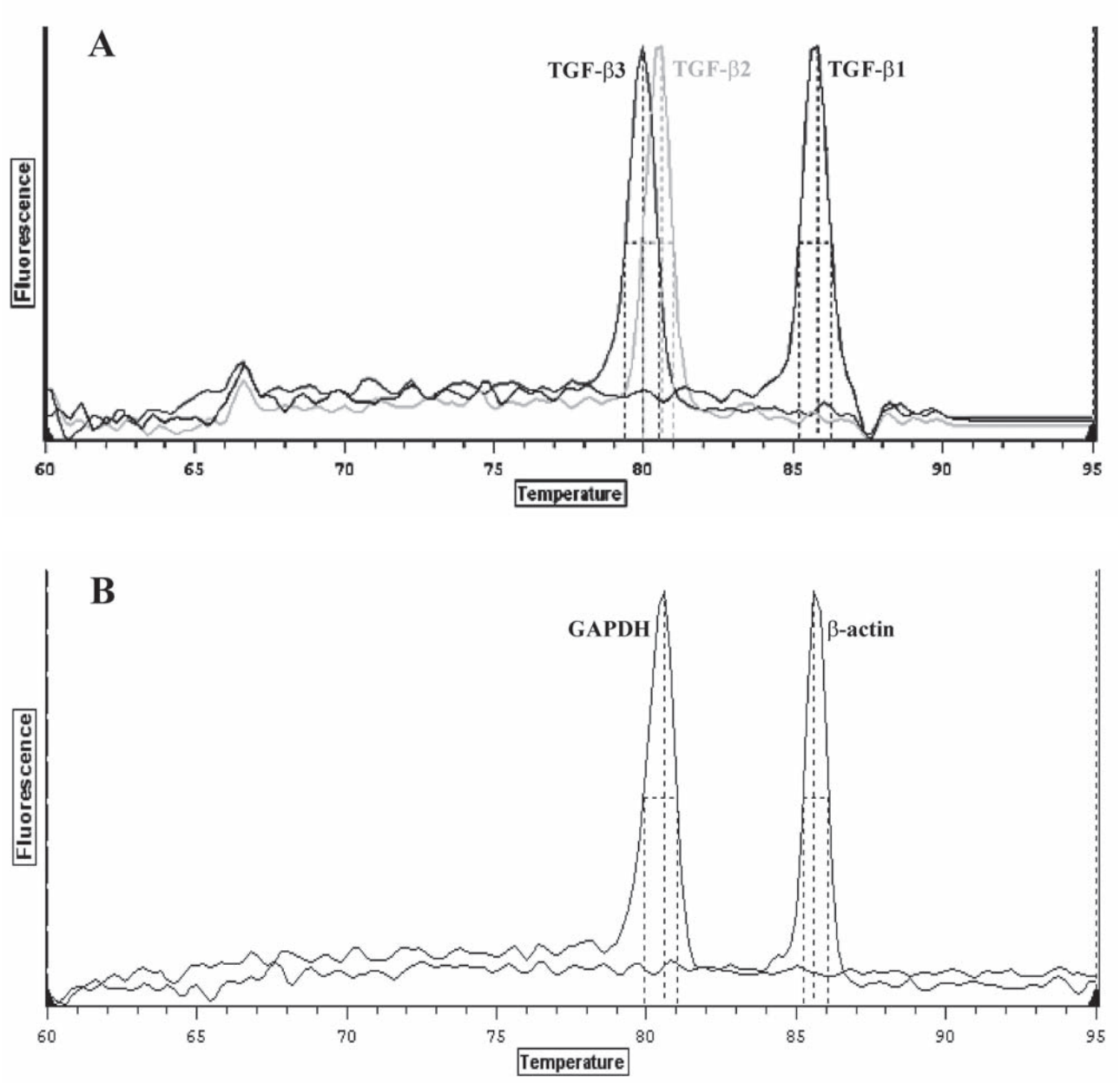

Fig. 1. Melting curve analysis of the PCR products of *TGF-β* isoforms and housekeeping genes (*GAPDH* and *β-actin*)**. A**: TGF-β1, TGF-β2, TGF-β3. **B**: GAPDH, β-actin.

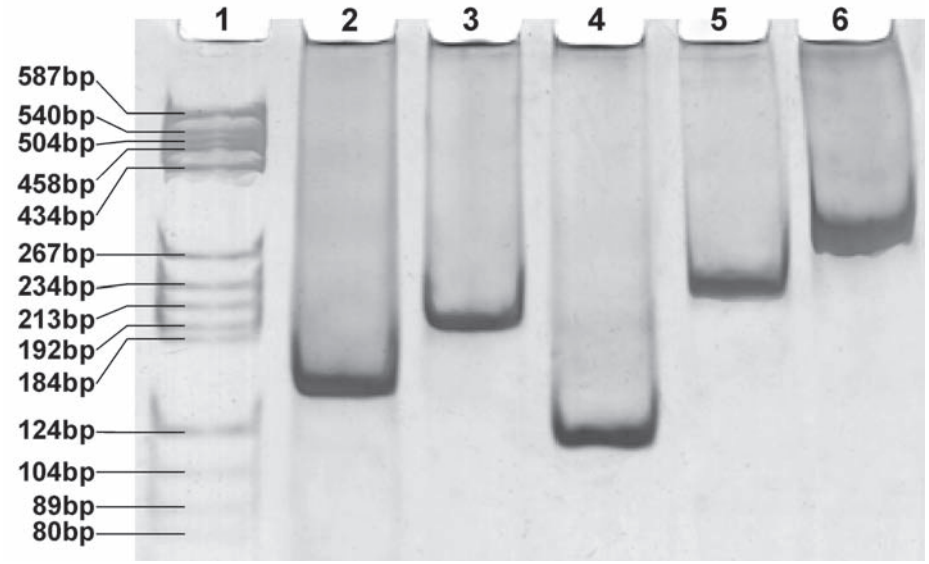

Fig. 2. RT-PCR products on 6% PAA electrophoresis; lane 1 – marker of size pBR 322/*Hae*III, lane 2 – TGF-β1 (151 bp); lane 3 – TGF-β2 (201 bp); lane 4 – TGF-β3 (121 bp); lane 5 – GAPDH (226 bp); lane 6 – β-actin (295 bp).

were demonstrated. The level of β-actin mRNA (median = 12750 copies/μg RNA) was higher than in the case of GAPDH (median = 2670 copies/μg RNA) and lower dispersion of the results for β-actin was noted (coefficient of variation: Cv = 20.85 for GAPDH, Cv = 9.90 for β-actin) (Fig. 3).

In the next step of the study we assessed TGF-β1, 2, 3 mRNA levels in normal corneal epithelium and after that we evaluated the quantitative relations between mRNA of these three isoforms. TGF-β1, 2, 3 isoforms were present in all tested samples obtained from corneal epithelium (TGF-β1: median = 3455 copies/μg RNA, TGF-β2: median = 473 copies/μg RNA, TGF-β3: median = 3520 copies/μg RNA). Comparative analysis of all TGF-β mRNA copies/1 μg of total RNA for each isoform showed that: TGF-β1 > TGF-β2; TGF-β3 > TGF-β2; TGF-β1 = TGF-β3 (post-hoc Tukey's test: TGF-β1 and TGF-β2, P = 0.0306; TGF-β3 and TGF-β2, P = 0.0045; TGF-β1 and TGF-β3 NS) (Fig. 4). Statistically significant differences in the transcriptional activity of TGF-β1 and TGF-β3 were not observed. Additionally, lower dispersion of the results for the TGF-β3 isoform was confirmed (coefficient of variation: Cv = 26.44 for TGF-β1; Cv = 10.53 for TGF-β3).

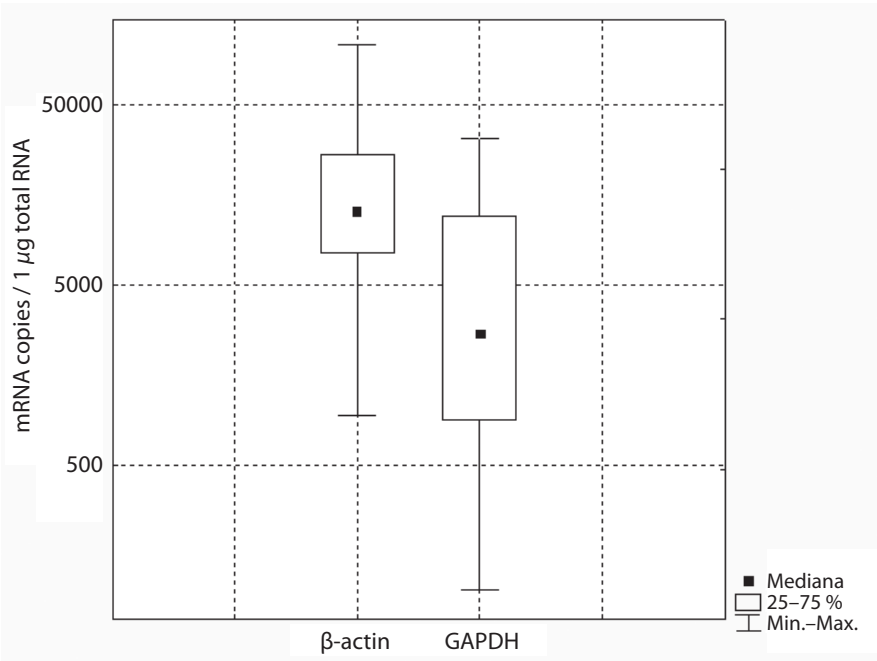

Fig. 3. GAPDH and *β-actin* gene expression in normal corneal epithelium

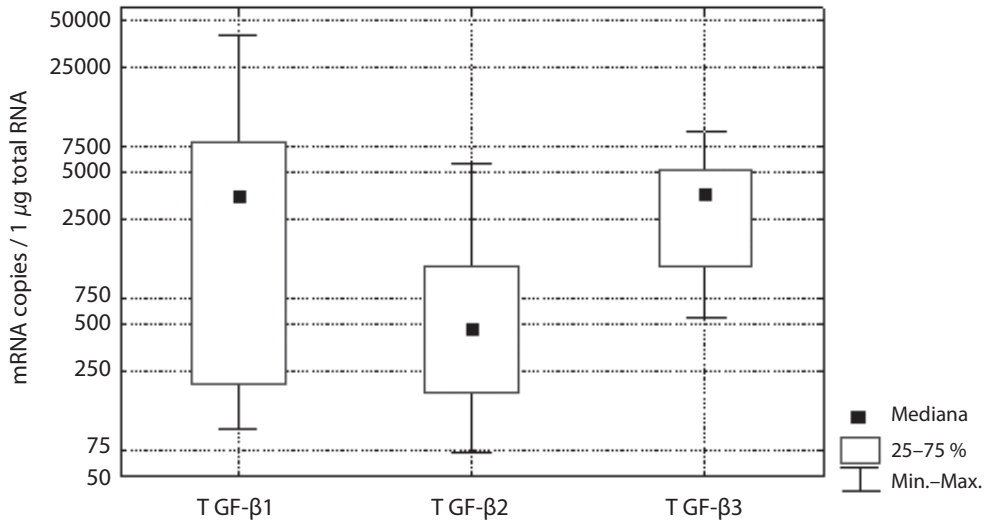

Fig. 4. Comparison of *TGF-β1*, *TGF-β2* and *TGF-β3* gene expression in normal corneal epithelium.

Discussion

The role played by TGF-β1, -2, and -3 isoforms in the cornea has been relatively well investigated (Chen et al., 2000; Hayashida-Hibino and Watanabe, 2001), but the quantitative relations between mRNA of these three isoforms still remain unknown. Although there are many reports concerning evaluation of the expression of TGF-βs in pathological changes in the cornea (Zieske et al., 2001; Song et al., 2002), only in several studies the gene expression profile of these cytokines in normal eye tissue was assessed (Li et al., 1999; Hayashida-Hibino and Watanabe, 2001). To our knowledge, this is the only one of the few reports concerning quantitative identification of the three TGF-β isoforms mRNA simultaneously in human corneal epithelium.

Analysis of gene expression in the eye has been notoriously difficult because of the technical obstacles associated with extracting sufficient quantities of high-quality RNA from the tissues. This is especially true for the lens and cornea, which have relatively few RNA-producing cells to compare with a highly cellular tissue such as retina (Diehn et al., 2005).

In the current work we used the SYBR Green-based real-time quantitative PCR procedure to analyse the gene expression profile of the three mentioned TGF-β isoforms in the human corneal epithelium. This technique enables simultaneous measurement of gene expression in many different samples for a limited number of genes (Bustin, 2000) and is more reproducible and sensitive than conventional quantitative methods. Furthermore, in all samples tested, mRNA of GAPDH and mRNA of β-actin were detected, which indicated the integrity of the RNA extracts and lack of RT-PCR inhibitors. In the final expression data, the studied genes were recalculated per 1 μg total RNA as recommended by Bustin (2002) and Tricarico et al. (2002), especially as higher dispersion of the results for both internal controls was noted.

We found mRNA expression of all the three TGF-β isoforms in human corneal epithelium. Our results are in agreement with previously published data (Millan et al., 1991; Pasquale et al., 1993; Hales et al., 1995); however, the mammalian eye then constituted the examined materials. Moreover, these reports affected only evaluation of the TGF-β presence without giving quantitative relationships between individual isoforms. Current data are also partly convergent with observations of Mita et al. (1998) and Chen et al. (2000), whose immunohistochemical studies revealed the presence or localization, but not the expression, of all the three TGF-βs in the regenerating epithelial cells of rats. Unfortunately, these studies have not been performed in human tissues. Li et al. (1999) showed that the TGF-β family is differentially expressed and regulated in cultured human corneal, limbal and conjunctival fibroblasts, which is consistent with our studies. Only a minor part of previous reports showed quantitative relationships between particular TGF-β isoforms in normal corneal tissue. E.g. Chen et al. (2000) detected substantial levels of the latent TGF-β1 (38 ng/ml) and TGF-β2 (2 ng/ml), probably originating from the lacrimal gland epithelial cells, in tears of normal rats. Additionally, they have shown that the levels of TGF-β2 and TGF-β3 mRNAs varied significantly after excimer ablation. A very low TGF-β2 mRNAs level (0.1 copy/cell), as it was determined, entirely confirmed our observation for human corneal tissue. Moreover, Cordeiro (2003) in studies concentrating on expression of the three TGF-β isoforms in a mouse model showed that all of them were present in the eye at the mRNA and/or protein levels, but TGF-β2 seemed to be the predominant isoform both in normal eyes and during the conjunctival wound-healing response. We did not find any report presenting transcriptional activity of TGF- β2 in normal corneal epithelium.

Comparative analysis of all TGF-β mRNA copies per 1 μg of total RNA for each isoform performed in the

current report showed that TGF-β1 and TGF-β3 were dominant isoforms in human corneal epithelium. Our observation is supported by data published by Carrington et al. (2006), who confirmed that TGF-β1 is the principal isoform in bovine corneal wound healing and suggested that inhibition of its action can promote corneal wound healing. Hayashida-Hibino and Watanabe (2001), by using cDNA expression array, established the effect of TGF-β1 on differential gene expression profiles in human corneal epithelium. Their observations indicate that TGF-β1 may control the differentiation and proliferation of corneal epithelial cells through changing expression levels of specific genes.

In summary, we found different expression of the TGF-β1, -2 and -3 isoforms in the human corneal epithelium. Such differential expression of TGF-β isoforms suggests that each of them may play a specific role in corneal tissue.

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