Original Article

Posttraumatic Temporal TGF-β mRNA Expression in Lens Epithelial Cells of Paediatric Patients

(traumatic cataract / anterior lens capsule / mRNA / TGF-β)

P. BEREZOWSKI[1], B. STRZALKA-MROZIK[2], M. FORMINSKA-KAPUSCIK[1], U. MAZUREK[2], E. FILIPEK[1], L. NAWROCKA[1], E. PIECZARA[1], P. BANASIAK[1], M. KIMSA[2]

[1]Division of Pediatric Ophthalmology, Department of Ophthalmology, Medical University of Silesia, Katowice, Poland
[2]Department of Molecular Biology, Medical University of Silesia, Sosnowiec, Poland

Abstract. The aim of the study was to determine temporal *TGFB1*, *TGFB2* and *TGFB3* gene expression profiles in the anterior lens capsule of paediatric patients with posttraumatic cataract. The patient group comprised 22 children selected with a fragment of anterior lens capsule obtained during elective cataract surgery and sampled for molecular analysis. The levels of TGF-β isoforms in the anterior lens capsule were determined based on the number of mRNA copies per 1 µg total RNA by real-time qRT-PCR. Three time-related result clusters were identified based on hierarchical cluster analysis: 2.2, 4.4 and 15.0 months (time span from injury to anterior capsule sampling during surgery) and compared with regard to temporal gene expression profile and quantitative relations of TGF-β1, 2 and 3 mRNAs. TGF-β1, TGF-β2, and TGF-β3 mRNAs were detected in all anterior lens capsule samples. A comparative analysis revealed: TGF-β1>TGF-β2>TGF-β3 during the entire observation period. The TGF-β mRNA levels continued to increase up to four months after injury, then returning close to the base levels after around 15 months. The expression patterns of TGF-β isoforms showed a similar tendency. Differences in the expression levels of TGF-β1 and TGF-β2 between the particular clusters were statistically significant. Posttraumatic transcriptional activities of TGF-β1 and TGF-β2 in the anterior lens capsule of paediatric patients depend on the time elapsing from injury. Our findings indicate that the transcriptional activities of *TGFB* family genes show a transient period of over-expression during the months after injury. TGF-β1 is a dominant isoform expressed in lens epithelial cells following injury.

Introduction

Lens opacification is the most frequent complication after perforating eye injury (Beby et al., 2006). Approximately 30 % of paediatric patients after surgical repair for perforating eye injury develop posttraumatic cataract and require repeated surgery (Dannenberg et al., 1992; Beby et al., 2006). It is estimated that trauma accounts for 6.9 % of lens opacities (Doutetien et al., 2008). Traumatic ocular emergencies are mostly observed in young patients, 40 % of which are children below the age of 10. Boys sustain perforating eye injury three times more often than girls (Beby et al., 2006; Doutetien et al., 2008). Generally, opacification affects the complete lens and is usually unilateral (Dannenberg et al., 1992; Beby et al., 2006; Doutetien et al., 2008). The treatment involves surgical removal of the lens; however, postoperative aphakia or pseudophakia and amblyopia treatment pose problems (Meacock et al., 2000; Strzalka-Mrozik et al., 2010). The mechanisms underlying posttraumatic lens opacification remain unclear. Several investigations have suggested a leading role of epithelial-mesenchymal transition (EMT), a process whereby lens epithelial cells undergo transition into a mesenchymal phenotype resulting in transparency loss and changes in lens fibre cells (Saika et al., 2000; de Iongh et al., 2001; Lovicu et al., 2002; Robertson et al., 2007; Xiao et al., 2009). Growth factors, especially the transforming growth factor β family (TGF-β), are implicated in the EMT of various epithelial tissues, including

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Corresponding author: Piotr Berezowski, Dąbrowskiego 3/2 Street, 43-300 Bielsko-Biała, Poland. Phone: (+48) 606 960 790; e-mail: berezbba@gmail.com

Abbreviations: ALC – anterior lens capsules, Ct – cycle threshold, EMT – epithelial-mesenchymal transition, GAPDH – glyceraldehyde-3-phosphate dehydrogenase, LEC – lens epithelial cells, qRT-PCR – quantitative real-time PCR, TGF-β – transforming growth factor β.

lens epithelial cells (LEC), in response to injury (Gordon-Thomson et al., 1998; Wormstone et al., 2002; Saika et al., 2003, 2004). In humans, three TGF-β isoforms (TGF-β1, TGF-β2, TGF-β3), which play roles in many physiological and pathological processes, are secreted (Saika, 2006).

TGF-β isoforms consist of regulators of developmental and homeostatic processes including cell and tissue differentiation, organogenesis, and maintenance of tissue and organ homeostasis (Dünker and Krieglstein, 2000; Moustakas et al., 2002). TGF-β1 stimulated fibroblast differentiation and increased secretion of collagen and fibronectin, thereby actively participating in reparative processes. TGF-β2 plays an essential role in cell growth, extracellular matrix production, epithelial-mesenchymal transition, and tissue remodelling. TGF-β3 controls regulation of molecules involved in cellular adhesion and ECM formation. TGF-βs are also important mediators of fibrogenic responses and thus act as potent stimulants of scarring, apoptosis, and neovascularization (Verrecchia and Mauviel, 2002; Schiller et al., 2004; Saika, 2006; Wormstone et al., 2006).

Previous study in human ocular tissues demonstrated that TGF-β may induce cataract formation (Gordon-Thomson et al., 1998; Meacock et al., 2000; Lois et al., 2003). TGF-β has also been implicated in other various trauma-related ocular pathologies (Kon et al., 1999; Picht et al., 2001; Schlötzer-Schrehardt et al., 2001; Saika, 2004a; Gacka and Adamiec, 2006; Wordinger et al., 2007). Although there are many reports concerning evaluation of the role of TGF-βs in pathological changes in the eyes (Zieske et al., 2001; Song et al., 2002), only in a few studies the level of these cytokines in normal eye tissue was assessed (Li et al., 1999; Hayashida-Hibino and Watanabe, 2001). The normal level of TGF-β in the aqueous environment may influence inhibition of cell proliferation in the central lens epithelium (Kurosaka and Nagamoto, 1994; de Iongh et al., 2005). Moreover, signalling via TGF-β receptors is required to maintain fibre cell survival (de Iongh et al., 2001; Lovicu et al., 2011).

The authors of most research reports determine only the level of TGF-β protein using different immunoenzymatic methods (Gordon-Thomson et al., 1998; Saika et al., 2000; Decologne et al., 2007). It should be noted that the change of protein level is preceded by alteration of transcriptional activity of the gene encoding this protein. There are, however, no published data regarding differences in mRNA levels of the three TGF-β isoforms after injury in human LEC. Therefore, the present study focuses on determining temporal changes in TGF-β1, TGF-β2 and TGF-β3 mRNA levels in LEC after injury in children.

Material and Methods

Patients

The study was approved by the Bioethics Committee of the Medical University in Katowice (KNW/0022/KB1/63/I/09) in accordance with the Declaration of Helsinki regarding medical research involving human subjects. The study and its purpose were explained to each participant or their legal guardian, who gave their informed written consent.

The patient group comprised 22 individuals (six girls and 16 boys, mean age 11.4; range 3.9 to 17.9 years) with a clinical diagnosis of traumatic cataract, treated in the Department of Ophthalmology, University Hospital No. 5, Medical University of Silesia, Katowice, Poland.

The diagnosis of traumatic cataract was based on the Birmingham Eye Trauma Terminology System of Kuhn et al. (2004). The mean time interval between injury and cataract surgery was 14.9 months (range 0.5 to 25.2 months). Three time-related result clusters (time span from injury to anterior capsule sampling during cataract surgery) were identified based on hierarchical cluster analysis: 2.2 months – 8 patients (cluster I), 4.4 months – 7 patients (cluster II) and 15.0 months – 7 patients (cluster III).

The criteria for inclusion in the molecular analysis were as follows: age ≤ 18 years, absence of general inflammatory conditions (prior to surgery all children were examined by a paediatrician, anaesthesiologist, ENT specialist and dentist) and systemic disease. We also ruled out local pathologies not related to injury.

Tissues

Circular sections of anterior lens capsules (ALC) with attached anterior lens epithelial cells (A-LEC) were obtained during cataract surgery and were stored for 48 h at -70 °C until RNA extraction.

RNA extraction from tissue specimens

Total RNA was extracted from ALC using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA extracts were treated with DNase I (MBI Fermentas, Vilnius, Lithuania), according to manufacturer's instructions. The quality of extracts was checked electrophoretically using 0.8% agarose gel stained with ethidium bromide. Results were analysed and recorded using the gel documentation system 1D Bas-Sys (Biotech-Fisher, Perth, Australia). Total RNA concentration was determined by spectrophotometric measurement in 5-µl capillary tubes using the Gene Quant II RNA/DNA Calculator (Pharmacia Biotech, Cambridge, UK).

Real-time qRT-PCR assay

Gene expression of *TGFB1, 2, 3* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes were evaluated using real-time qRT-PCR and SYBR Green I chemistry (SYBR Green Quantitect RT-PCR Kit, QIAGEN, Valencia, CA). The analysis was carried out using an Opticon™ DNA Engine Continuous Fluorescence Detector (MJ Research, Watertown, MA). All samples were tested in triplicate. *GAPDH* was included in order to monitor the qRT-PCR efficiency. Oligonucleotide primers specific for *TGFB1, 2, 3*, and *GAPDH* genes were described previously by Strzalka et al.

(2008) and Ercolani et al. (1988), respectively. The thermal profile for one-step RT-PCR was as follows: reverse transcription at 50 °C for 30 min, denaturation at 95 °C for 15 min, 50 cycles consisting of temperatures: 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. The point at which the PCR product is first detected above a fixed threshold – termed cycle threshold (Ct) – was determined for each sample, and the average Ct of triplicate samples was calculated. Each run was completed using melting curve analysis to confirm specificity of the amplification and absence of primer dimers. RT-PCR products were separated on 6% polyacrylamide gels and visualized with silver salts.

Quantification of expression of target genes

To quantify the results obtained by RT-PCR for *TGFB1, 2, 3* and *GAPDH*, the standard curve method was used (Strzalka-Mrozik et al., 2010). In order to simultaneously detect the expression profile of each investigated gene, commercially available standards of β-actin cDNA (TaqMan® DNA Template Reagent Kit, PE Applied Biosystems, Inc., Foster, CA) were used at five different concentrations (0.6, 1.2, 3.0, 6.0, and 12.0 ng/µl). Values of copy numbers for standards were calculated based on the relationship: 1 ng of DNA = 333 genome equivalents (PE Applied Biosystems). Amplification plots for each dilution of the commercially available standard template were used to determine Ct values. A standard curve was generated by plotting Ct values against the log of the known amount of β-actin cDNA copy numbers. Correlation coefficients for standard curves ranged from 0.988 to 0.995, indicating a high degree of confidence for measurement of the copy number of molecules in each sample. The copy numbers of analysed mRNAs were calculated from the linear regression of the standard curve.

Statistical analyses

Statistical analyses were performed using Statistica 8.0 software (StatSoft, Tulsa, OK); the level of significance was set at $P < 0.05$. Values are expressed as median (Me) with the 25th and 75th quartiles. Temporal expression profile of *TGFB1*, *TGFB2* and *TGFB3* genes was assessed using hierarchical cluster analysis. Following standardization, the variables were analysed with Ward's method and Euclidean distance. Three time-related result clusters were identified (at 2.2, 4.4 and 15.0 months of injury) and analysed using a non-hierarchical clustering algorithm (k-means), and, additionally, cross-validation. Observational values assigned to relevant clusters were then analysed with nonparametric Kruskal-Wallis test.

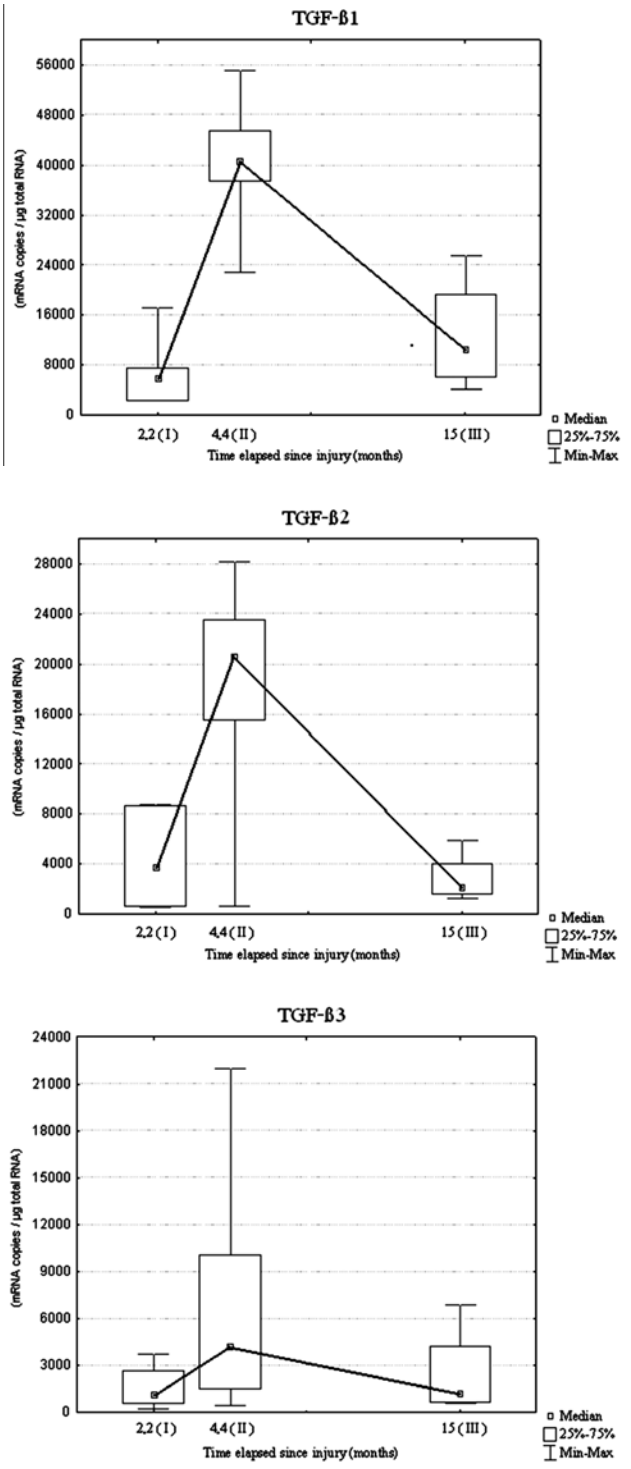

Fig. 1. Changes in the expression of TGF-β1, TGF-β2 and TGF-β3 (mRNA copies per 1 µg total RNA) in the anterior lens capsule over the 0.5 to 25 months after penetrating eye injury. Three time-related result clusters based on hierarchical cluster analysis: 2.2 months (cluster I), 4.4 months (cluster II) and 15.0 months (cluster III).

Results

Transcriptional activity of TGF-β isoforms in human anterior capsule explants was determined using real-time qRT-PCR. In the first step of the study, specificity of the RT-PCR assay for the target genes was confirmed experimentally on the basis of the amplimers' melting temperatures. For each RT-PCR product, a single peak at the expected temperature was observed: TGF-β1 85.4 °C; TGF-β2 80.0 °C; TGF-β3 80.6 °C; GAPDH 80.1 °C. Gel electrophoresis also revealed the presence

Table 1. The mRNA levels of TGF-β1, TGF-β2, TGF-β3 in the anterior lens capsule over the 0.5 to 25 months after penetrating eye injury

	Anterior lens capsule (ALC)					
	2.2 months (I)		**4.4 months (II)**		**15 months (III)**	**P***
TGF-β1 mRNA	5916 (2372-7547)	<	40716 (37399-45552)	>	10500 (6050-19258)	(P = 0.0032)
TGF-β2 mRNA	3727 (665-8633)	<	20598 (15534-23526)	>	2085 (1647-4030)	(P = 0.0502)
TGF-β3 mRNA	1167 (541-2674)	<	4178 (1498-10100)	>	1179 (628-4256)	(P = 0.12)
mRNA	TGF-β1 > TGF-β2 > TGF-β3		TGF-β1 > TGF-β2 > TGF-β3		TGF-β1 > TGF-β2 > TGF-β3	(P < 0.05)

Statistical significance: P < 0.05; P* Kruskal-Wallis test
Median with the 25th and 75th quartiles is presented (mRNA copies/μg total RNA)
Three time-related result clusters based on hierarchical cluster analysis: 2.2 months (cluster I), 4.4 months (cluster II) and 15.0 months (cluster III)

of a single product of the predicted length (data not shown).

In the next step, levels of TGF-β1, TGF-β2 and TGF-β3 mRNAs in human anterior capsule explants were assessed. TGF-β1, TGF-β2 and TGF-β3 isoforms were detected in all tested samples (TGF-β1 Me = 13061 copies/μg RNA; TGF-β2 Me = 5900 copies/μg RNA; TGF-β3 Me = 1468 copies/μg RNA). Then three time-related result clusters (time span from injury to anterior capsule sampling during cataract surgery) were identified based on hierarchical cluster analysis: 2.2 (cluster I), 4.4 (cluster II) and 15.0 months (cluster III). Clusters were compared with regard to temporal gene expression profile and quantitative relations of TGF-β1, 2 and 3 mRNAs (Fig. 1).

Comparative analysis of all TGF-β isoform mRNA copies/μg of total RNA revealed the following relationships in anterior lens capsule after injury: cluster I – TGF-β1>TGF-β2>TGF-β3 (P = 0.0031, Kruskal-Wallis test); cluster II – TGF-β1>TGF-β2>TGF-β3 (P = 0.0184, Kruskal-Wallis test); cluster III – TGF-β1>TGF-β2 >TGF-β3 (P = 0.0498, Kruskal-Wallis test) (Table 1).

Posttraumatic changes in *TGFB* gene expression within clusters under consideration were comparable for all three tested isoforms. TGF-β mRNA levels continued to increase up to four months after injury, then returning close to the baseline levels after around 15 months. The changes in expression were statistically significant for TGF-β1 (P = 0.003, Kruskal-Wallis test) and TGF-β2 (P = 0.05, Kruskal-Wallis test), while for the TGF-β3 isoform statistically significant alteration was not found (NS, Kruskal-Wallis test).

Discussion

TGF-β is among the most important factors involved in ocular physiology and pathology, mainly expressed in the ciliary epithelium, subcapsular epithelium and equatorial region of the lens (Klenkler and Sheardown, 2004; Yamamoto et al., 2005; Saika, 2006). Unfortunately, the quantitative relationships between mRNA expression of different TGF-β isoforms in posttraumatic anterior lens capsule of paediatric patients still remain unknown. In most studies the level of TGF-β isoforms in ocular tissues was determined using ELISA methods (Gordon-Thomson et al., 1998; Saika et al., 2000). Only in a few reports the transcriptional activity of TGF-βs was detected (Gordon-Thomson et al., 1998; Saika et al., 2000; Strzalka et al., 2008; Strzalka-Mrozik et al., 2010).

In our study the real-time qRT-PCR technique was used to evaluate TGF-β1, TGF-β2 and TGF-β3 mRNA levels in anterior lens capsules. Three TGF-β isoforms were detected in all samples obtained during cataract surgery. Gordon-Thomson et al. (1998) revealed the presence of TGF-β1, 2, 3 ligands in the anterior lens capsule. Moreover, the authors detected signals for TGF-β1 and TGF-β2 mRNAs by *in situ* hybridization. Interestingly and unlike in our investigations, the expression of mRNA for TGF-β3 was not detected.

Posterior capsule opacification strongly depended on the TGF-β signalling pathway, and commonly occured at 6 to 12 months of cataract surgery, similarly to peak TGF-β concentrations observed in our study (Lois et al., 2005; Bras et al., 2006). TGF-β1 was a predominant isoform in three time-related result clusters in ALC, which indicated a strong association with posttraumatic ocular conditions. Our observation is supported by data published by Li et al. (1999) and Carrington et al. (2006), who indicated the strongest expression of TGF-β1 mRNA, but in human corneal, limbal, conjunctival fibroblasts and bovine cornea, respectively. On the other hand, Shirai et al. (2006) performed studies where epithelial-mesenchymal transition lens cells expressed TGF-β1, TGF-β2, and TGF-β3 isoforms. Furthermore, the authors showed an increase in the amount of both active and total TGF-β2 in injured rat lens. Connor et al. (1989) and Saika (2004b) also suggested that the TGF-β2 isoform plays the most important role in ocular tissues and revealed that it is expressed at much higher levels than the other TGF-β isoforms both in the aqueous and in the vitreous humour.

Reports on animal and human lens epithelial cell line models support the cataractogenic potential of TGF-β (Gordon-Thomson et al., 1998; Hales et al., 1999); the ensuing lesions are, at the cellular level, indistinguishable from some forms of human cataract (Lovicu et al., 2002). Exogenous TGF-β1, 2 and 3 added to cultures of mouse lens epithelial cells induce formation of aberrant spindle-cells clumps, lens capsule wrinkling, apoptotic cell death of anterior lens epithelium, and extracellular

matrix accumulation resulting in the development of subcapsular opacities (Hales et al., 1995). However, TGF-β2 and TGF-β3 isoforms were ten times more potent contributors to the cataractous process when compared to exogenous TGF-β1 (Gordon-Thomson et al., 1998; Cerra et al., 2003). Elevated TGF-β1 levels also induce oedema, damage to subcapsular and equatorial cortical fibres, and migration of collagen-containing cells from the posterior capsule to the posterior pole of the lens (Flügel-Koch et al., 2002).

Saika et al. (2003, 2004) have demonstrated that penetrating eye injury causes nuclear translocation of Smads in lens epithelial cells, thereby documenting TGF-β signalling pathway activation following injury. Similar observations were noticed by Wallentin et al. (1998), who proved the activation of the TGF-β superfamily signalling pathway following experimental cataract surgery in rabbits. Moreover, in this study the continuously increasing level of total TGF-β in postoperative aqueous humour was performed. Interestingly, in our study the mRNA levels continued to increase up to four months after injury, then returning close to the baseline levels after around 15 months, which is partially consistent with the above-mentioned results.

Summary

Transcriptional activities of *TGFB1*, *TGFB2* and *TGFB3* genes in anterior lens capsule of children with traumatic cataract depends on the time that has elapsed since the injury. Transient *TGFB* over-expression in posttraumatic ALC, with peak concentrations at four months of injury, may indicate its involvement at later stages of the healing process.

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