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Fibrosis: Altered gene expression in TGF-β stimulated human fibroblasts of the Tenon

Abstract: Despite decades of research, fibrosis still remains a significant challenge for medicine in many different fields. Although there is a general model of fibrosis, the causes and characteristics of the various pathologies are as diverse as the variety of organs and tissues that can be affected by fibrosis. Moreover, fibrosis also impedes the long-term prospects of success in implantation surgery. One possibility to address this challenge is the development of biocompatible implants featuring drug delivery systems loaded with antifibrotic pharmaceuticals. Due to diverse regulatory mechanisms in organs, tissues and also cell types, these active substances must consequentially be designed for diverse specific applications. Compared to fibrosis in organs like lung or liver, these mechanisms were poorly addressed in ophthalmologic research, but it is known that transforming growth factor beta (TGF-β) plays a key role.

This gene expression study revealed 30 genes being upregulated more than two fold in TGF-β1 treated human primary tenon fibroblasts (hTF). Furthermore, 15 genes were found to be downregulated more than two fold. Tumor necrosis factor (*TNF*), vascular endothelial growth factor A (*VEGFA*) and inhibin beta (*INHBE*) were particular strongly regulated in TGF-β1 treated hTFs.

Keywords: fibrosis, ophthalmic implant, gene expression

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1 Introduction

Fibrosis is a complex process of pathological wound healing that leads to the loss of physiological integrity and function in various organs and tissues [1]. Especially in ophthalmology fibrosis is one of the main causes of postoperative complications and implant failure [2, 3].

Intensive research over the last 20 years has led to a general concept of the molecular biological mechanisms in fibrosis development. Basically, the activation of TGF-β receptors by the cytokine TGF-β leads, via a complex cellular network, to an increased expression of proteins of the extra cellular matrix (ECM) and to the inhibition of matrix-regulating factors [4]. The ECM is defined as a non-cellular three-dimensional macromolecular network composed of collagens, elastin, fibronectin, laminins, and several other proteins and glycoproteins [5]. Myofibroblasts which derive from fibroblasts were the primary collagen-producing cells in this pathological process [6]. In turn these fibroblasts not only differ among organs, they also display heterogenous phenotypes within single organs [7].

From the eye, fibroblast subpopulations of four different tissues (Tenon capsule, sclera, chorioidea and orbital fat) have been reported so far by STAHNKE et al. [8]. Moreover primary fibroblasts from these tissues were isolated and used for the development of a fibrosis model to predict pharmacological efficacy of antifibrotic pharmaca [9, 10].

This study is a first insight into a molecular characterisation of the previously described fibroblast subpopulations via real-time quantitative PCR of 84 different fibrosis related genes. As a first approach, this study focused on the induction of the gene expression in hTFs after stimulation with TGF-β1. On the one hand specific biomarkers might be identified to improve the characterisation of fibrotic processes in the eye, on the other hand targets which can be inhibited by alternative antifibrotic pharmaceuticals might be identified.

2 Materials and methods

2.1 Cell culture and TGF-β treatment

Human primary fibroblasts from the Tenon capsule were isolated as described previously by STAHNKE et al. [8]. Collection of donor material and its further use was approved by the ethics committee of the University of Rostock (approval ID: A2011 11) and followed the guidelines of the Declaration of Helsinki. Cultivation of hTFs was carried out

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in Dulbecco's Modified Eagle Medium (DMEM) - low glucose (MERCK, Darmstadt, Germany) with 1 mg glucose, 1 % Penicillin/Streptomycin, 10 % fetal calf serum (FCS) and 3.7 g/L $NaHCO_3$ under standard cell culture conditions (37 °C, 5 % CO_2).

For the TGFβ-1 treatment hTFs were seeded into two cell culture flasks and incubated until 70 % confluence in DMEM with FCS. Before treatment, cells were incubated for 24 h in DMEM without FCS. Following this starving medium was removed and DMEM containing 10 ng/mL TGF-β1 was given to one of the flasks. The same procedure without TGF-β1 was performed on the second flask which served as control.

2.2 Isolation of ribonucleic acids

For Isolation of the ribonucleic acids (RNA) 2 mL TRIZOL reagent (Ambion) was given to the cell culture flasks. Suspension with disrupted cell material was then transferred into two 1.5mL tubes and covered with chloroform (100 µL) and □-mercaptoehtanol (10 µL). After mixing and centrifugation supernatant was transferred to a new 1.5 mL tube and covered with Trizol (400 µL), chloroform : isoamyl alcohol (40 µL) and □-mercaptoehtanol (4 µL). Further purification of RNA was conducted with RNeasy mini kit (Qiagen, Hilden, Germany) including a DNAseI digestion.

2.3 cDNA synthesis and qRT-PCR

Synthesis of cDNA was conducted with RT² First strand kit (Qiagen). A RNA amount 0.5 µg was given to the RT mix and incubated in a thermos cycler (42°C for 15 min and subsequent 95°C for 95 min).

RT-qPCR was carried out with RT² Profiler PCR Array for human fibrosis (Qiagen) in Eppendorf realplex² Mastercycler. PCR mix containing cDNA was directly pipetted into the 96 wells of the RT² Profiler PCR plate prepared from the manufacturer. Beside fibrosis related genes 5 housekeeping genes were detected to normalise Ct values. For information regarding the plate layout and full gene names please visit Qiagen homepage.

Ct values were first normalised with geometric mean of the housekeeping genes *ACTB, B2M, GAPDH, HPRT1* and *RPLP0*. TGF-β1 treatment group was then normalised with the untreated control to obtain relative transcription (□□Ct). Fold changes were subsequent calculated as described from PFAFFL 2004 [11].

3 Results and Discussion

Initially the evaluated fibrosis related genes were classified into functional groups, to simplify the overall comprehensibility of the data received. The PCR array provides measurement of various cytokines, transcription factors, growth factors, regulatory proteins, proteins of signal transduction, ECM components and remodelling enzymes.

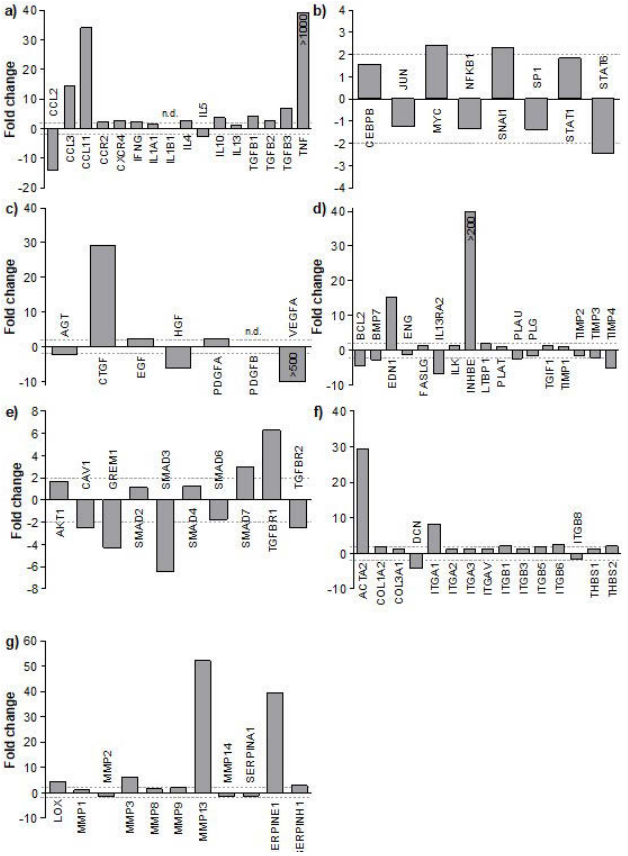

Figure 1: Fold changes of mRNA transcription obtained via qRT-PCR, calculated as previously described [11]. **a)** cytokines, **b)** transcription factors, **c)** growth factors, **d)** regulatory proteins, **e)** signal transduction proteins, **f)** ECM components, **g)** remodelling enzymes. Dashed lines indicate a fold change of 2.

In all functional groups we found remarkable alterations of the mRNA transcription in TGF-β1 treated hTFs. We found 30 genes being upregulated more than 2 fold and 15 genes being downregulated to the same extend.

3.1 Cytokines

Within the cytokines the chemokine CC motif ligands (CCLs) might play an important role in fibrogenesis. While CCL2 (-14.4 fold) is involved in the recruitment of immune cells like monocytes and basophiles [12], CCL3 (14.4 fold) is responsible for neutrophil [13], and CCL11 (34.1 fold) for

eosinophil [14] recruitment. TGF-□1 treated hTFs appear to set rather priority on phagocytising neutrophils and reactive oxygen species releasing eosinophils then on inflammation inducing basophiles.

TNF-mRNA (>1000 fold), which was near the detection limit in untreated hTFs was strongly induced. Beside its primary role in the regulation of immune cells, the multifunctional cytokine is also responsible for the induction of apoptosis [15, 16]. Upregulation of TNF might here compensate exuberant cell growth. Moreover myofibroblasts undergo apoptosis in physiological woundhealing

3.2 Transcription factors

In contrast to cytokines the transcription factors were poorly regulated in the TGF-β1 treated hTFs. Nevertheless we found transcription factor *MYC* (2.4 fold) and *SNAI1* (2.3 fold) upregulated while *STAT6* (-2.4 fold) was downregulated.
MYC is a general amplifier of gene expression and implicated as well with physiological as with pathological cell growth, proliferation, metabolism, and differentiation [17, 18]. Upregulation of *MYC* might be interpreted as marker function for cells with enhanced activity.

The similarly upregulated *SNAI1* is a highly specific transcription factor. It is a repressor of the adhesion molecule E-cadherin, which promotes cell adhesion and is downregulated in epithelial mesenchymal transition [19].
STAT6 expression is known to be induced by *IL4* [20], which we also found upregulated in TGF-β1 treated hTFs (2.8 fold). This is an indicator for the involvement of the YAK-STAT signalling pathway, which induces genes regarding apoptosis and cell cycle.

3.3 Growth factors

The growth factor *CTGF* (29.3 fold) which we also found strongly induced is undoubtedly a key player in physiologic control of cell proliferation, differentiation, and adhesion. Moreover it is critically involved in tissue fibrosis [21].
VEGFA (> -500 fold) is a growth factor responsible for physiological but also pathological angiogenesis, which is known to be accompanied by tissue fibrosis [22]. Its downregulation in hTFs must remain undetermined.

3.4 Regulatory Proteins

The upregulated *EDN1* (15.3 fold) is involved in physiological wound healing but also drives fibroblast activation, proliferation, and myofibroblast differentiation in tissue fibrosis [23].

Upregulation of *INHBE* (>223.2 fold) is known to occur when unfolded and misfolded proteins accumulate in the endoplasmic reticulum of human fibroblasts [24]. This “ER stress” may result from overwhelming activation of cellular processes via TGF-β1.

3.5 Signal transduction proteins

The smad family members (*SMADs*) are described as key signalling factors of the TGF-β/SMAD signalling pathway [25]. *SMAD3* (-6.4 fold), which we found remarkably downregulated is on the one hand directly involved into the TGF-β induced transcription [26], but it is on the other hand triggering the transcriptional repression of *MYC* induced transcription [27].

The TGF-β receptors (*TGFBRs*) 1 and 2 build a heteromeric complex to bind TGF-β and to induce its downstream signalling pathway [28]. While *TGFBR1* (6.29 fold) was upregulated the *TGFBR2* (-2.5 fold) was found to be downregulated. Considering that only a stoichiometric increase in the expression of both receptors resulted in an amplification of the TGF-β1 signal [29], *TGFBR2* independent function might be considered here.

3.6 ECM components

The upregulation of the myofibroblast marker *ACTA2* (29.5 fold) [30] is perfectly evidencing the differentiation of hTFs to the myofibroblast cell-type in our fibrotic cell culture model.

Integrins are a complex group of transmembrane receptors that mediate ECM adhesion to cell membrane [31]. *ITGA1* (8.3), binding collagens, and laminins [32], was the only representative of the integrins, being remarkably upregulated.

3.7 Remodelling enzymes

Matrix metalloproteinases (*MMPs*) are a family of endopeptidases, which are collectively degrading all components of the ECM [33]. *MMP13* is activated by various other *MMPs* and has a central role in the formation and remodelling of bone substance [34]. The strong upregulation of *MMP13* (52.1 fold) suggests its crucial role in remodelling of ECM in TGF-β1 treated hTFs.
SERPINE1 (39.5 fold), which was strongly upregulated, regulates tissue homeostasis and wound healing by inhibiting plasmin-mediated *MMP* activation [35]. In models of liver, lung and kidney *SERPINE1* deficiency attenuates fibrosis [35].

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