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Nintedanib suppresses expression of extracellular matrix proteins in TGF- β 1-stimulated primary human fibroblasts of the Tenon

Abstract: The development of strategies for fibrosis prevention is a perpetual cutting the edge challenge, especially in ophthalmologic fistulating surgery. Stenosis of liquid draining implants but also fibrotic activation of implant-associated tissues are major clinical examples for fibrosis induced implant failure. Implant coating or incorporation of antifibrotic agents offer a promising approach to minimise failure rates. Nintedanib is a drug that has been successfully used for the treatment of idiopathic pulmonary fibrosis since 2015. In this study, we evaluated the suitability of Nintedanib for ophthalmic therapeutic treatment. Therefore, the antifibrotic potential of the active substance was tested with a fibrotic cell culture model on primary fibroblasts of the Tenon (hTF) *in vitro*.

The concentration of 10 μ M Nintedanib demonstrated a marginal effect on cell viability but coincidentally diminished cell proliferation remarkably. Both, immunocytochemical and Western blot analyses revealed a significant inhibitory effect of Nintedanib on the TGF- β 1 induced expression of the extracellular matrix (ECM) components fibronectin and collagen. Moreover it suppressed the expression and formation of stress fibres of the fibrotic marker protein alpha smooth muscle actin (α -SMA).

Keywords: fibrosis, glaucoma, ophthalmic implant, extracellular matrix, protein expression

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1 Introduction

Especially in ophthalmic implant surgery, fibrosis is still one of the main causes for postoperative complications [1]. Trabeculectomy [2] and implantation of glaucoma drainage implants [3] are only two examples for relevant surgical procedures that might undergo postoperative failure caused by fibrosis. Within the last decade an increasing number of ophthalmic implants were developed as drug delivery system to release pharmacological substances [4,5,6].

At the cellular level fibrosis is mainly characterised by increased cell proliferation as well as by overexpression of proteins of the extracellular matrix [7]. A fibrotic cell culture model established from Stahnke et al. [8] makes use of this aspect, to evaluate the efficacy of antifibrotic agents for ophthalmic indications. Nintedanib is a pharmaceutical substance that has been used successfully since 2015 for the treatment of idiopathic pulmonary fibrosis (IPF) [9]. The small molecule functions as a tyrosine kinase inhibitor and interferes with the fibroblast growth factor receptor (FGFR), which is mediating the pro-fibrotic pathways [10]. Pathway studies, reviewed by Shi and Chen [11] revealed complex interplays of TGF- β signalling and receptor tyrosine kinases such as FGFR. Moreover in IPF patients blocking of the receptor resulted in the inhibition of collagen I, V, and fibronectin expression as well as to a reduction of collagen I and III secretion [12]. These cell- and molecular biological correlations highlight Nintedanib as a potential therapeutic alternative, to prevent fibrotic processes in the ophthalmic field.

This study is intended to analyse the influence of the pharmaceutical Nintedanib on the described fibrotic processes in TGF- β 1-stimulated hTFs, considering the important ECM components and fibrotic markers in two different independent protein detection assays.

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2 Materials and methods

2.1 Cell culture, cell viability and proliferation

Human primary fibroblasts from the Tenon capsule were isolated as described previously by Stahnke et al. [13]. 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 in Dulbecco's Modified Eagle Medium (DMEM) - low glucose (MERCK, Darmstadt, Germany) with 1mg glucose, 1% Penicillin/Streptomycin, 10% fetal calf serum (FCS) and 3.7g/L NaHCO₃ under standard cell culture conditions (37°C, 5% CO₂).

Cell viability and proliferation assays were performed to determine biological active and tolerable concentrations of Nintedanib. For both tests 2x10⁴ hTFs were seeded into a 96-well microtiter plate with 200 μ L culture medium per well and incubated under cell culture conditions for 24h. After pre-incubation a dilution series of Nintedanib from 100 μ M to 1pM was prepared and given to the cells. Each concentration was tested in quadruplicate.

After 48h of incubation cell viability was tested with CellQuanti-Blue Cell Viability Assay Kit (CQB, BioAssay systems, Hayward, CA, USA), cell proliferation with Cell Proliferation ELISA (BrdU, Roche Diagnostics, Mannheim, Germany). While CQB assay is an indicator for the metabolic turnover of the cells, BrdU assay can provide insights into cell proliferation rates. Both assays were carried out after manufacturer instructions. Luminescence (CQB fluorescence and BrdU chemiluminescence) were measured with the Fluostar optima (BMG LABTECH, Ortenberg, Germany).

2.2 TGF- β 1 treatment and Western Blot

For the TGF- β 1 treatment hTFs were seeded into eight cell culture flasks and incubated until 70% confluence in DMEM with FCS. Before treatment, cells were incubated for 24h in DMEM without FCS. Following this, starving medium was removed and DMEM containing treatment reagents was given to the flasks according the scheme described in table 1. For Western Blot analysis treated hTFs were washed in PBS and subsequently lysed in sample buffer (Laemmli) containing 1% SDS.

Table 1: Scheme of hTF treatment for Western Blot

Flask/lane	10nM TGF- β 1	Nintedanib
1	-	-
2	-	10 μ M
3	-	1 μ M
4	-	100nM
5	+	-
6	+	10 μ M
7	+	1 μ M
8	+	100nM

Samples were boiled for 10min and protein content was determined according to Neuhoff et al. [14] after cooling on ice. For SDS-PAGE 10-30 μ g protein per lane was loaded in 7.5% or 10% polyacrylamide gels dependent on molecular weight. Polyacrylamide gels with the separated protein fractions were transferred to PVDF membranes (0.2 μ m, BIO-RAD, Munich, Germany). Free protein binding sites on the membranes were blocked with 5% nonfat dry milk powder in TRIS buffered saline (TBS) for 30min. Incubation with specific primary antibodies was then carried out at 4°C over night. Following antibodies were used (dilutions are given in brackets): mouse monoclonal anti-collagen I and VI (1:500), mouse monoclonal anti-fibronectin (1:1,000), mouse monoclonal anti- β -tubulin (1:1,000) and mouse monoclonal anti- α -SMA (1:500). Incubation of the membranes was conducted with secondary HRP-conjugated anti-mouse (1:2,500) IgG after three washing steps with TBS including Tween 20. Detection of secondary antibodies was achieved by the enhanced chemiluminescence (ECL) procedure as described by the manufacturer (Thermo scientific, Pierce, Rockford, USA).

2.3 Immunocytochemical analysis

For immunocytochemical analysis hTFs were cultivated on cover slips for 24h until the cells reached subconfluency. Treatment procedure with TGF- β 1 and Nintedanib was realised according to point 2.2. Subsequently to 48h incubation the coverslips were washed with PBS and fixated with 3% paraformaldehyde (PFA) for 10min. To ensure optimal intracellular protein staining, cells were pretreated with 0.1% Triton X-100 containing 2% FCS (30min) for permeabilisation of the cell membranes. Incubation with a 1:100 dilution of the mouse monoclonal anti- α -SMA antibody was carried out in PBS for 60min. In progress to the

incubation with the secondary antibody the cells were washed in PBS three times. The donkey anti-mouse IgG (H+L)-Alexa Fluor 488 secondary antibody was used at a 1:50 dilution. For the removal of secondary antibodies, cells were again rinsed three times with PBS and mounted. Unspecific staining was precluded with control experiments, using the secondary antibodies only. Staining of nuclei was conducted with 4, 6-diamidino-2-phenylindole (DAPI) (1μg/ml), which was included in the mounting medium (Vectashield, Vector Laboratories LTD, Peterborough, UK). Fluorescent labelling was analysed using Axioskop 40 (Zeiss, Jena, Germany) equipped with microscope light HBO 50 (Zeiss), Colibri 7 LED solid-state light source (Zeiss) and Axiovision software (version 4.8.1.; Zeiss) using the same settings.

3 Results

3.1 Cell viability and Proliferation

CQB - Assay was implemented to determine a biocompatible concentration of Nintedanib that should not be exceeded in further potential animal trials. According to the DIN EN ISO 10993 standard, a substance is biocompatible *in vitro* if the relative cell viability is greater than or equal to 70%. In parallel, the BrdU - Assay was conducted to ensure that cell proliferation was inhibited at this concentration.

Figure 1: Relative Cell Viability and Proliferation of hTFs treated with a dilution series of Nintedanib

At a concentration of 10μM Nintedanib we found a relative cell viability of 87.4%, while proliferation was diminished to 24.3% (Fig. 1).

3.2 Western Blot

For Western Blot analysis concentrations from 10μM to 100nM Nintedanib were chosen to cover the biocompatible range.

Western Blot revealed a significant inhibitory effect of Nintedanib on the TGF-β1 induced expression of the extracellular matrix (ECM) components fibronectin, collagen I, collagen VI as well as on α-SMA at a concentration of 10μM (Fig 2).

Figure 2: Western Blot from lysates of differently treated hTFs.

Even at a concentration of 1μM Nintedanib, the expression of the fibrosis markers fibronectin and α-SMA was attenuated. While expression of collagen I and VI was only slightly inhibited at a concentration of 10μM Nintedanib, fibronectin was clearly diminished and α-SMA expression was completely interrupted.

3.3 Immunocytochemical analysis

The excellent capability of Nintedanib to suppress fibronectin and α-SMA expression was further evaluated with immunocytochemical analysis. Microscopic imaging (Fig. 3) confirmed the Western Blot observations strikingly. All tested concentrations (10μM, 1μM and 100nM; data only shown for 1μM concentration) Nintedanib prohibited TGF-β1 treated hTFS from fibronectin expression as well as from formation of the fibrosis typical α-SMA stress fibers. While the concentration of 100nM had a convincing influence on α-SMA expression, the concentration of 10μM and 1μM led to total termination of stress fiber formation.

Figure 3: Microscopic imaging of differently treated hTFs. Fibronectin (red), α -SMA stress fibers (green), nuclei (blue)

4 Discussion

Both, the results from the Western Blot and the immunocytochemical analysis, highlight Nintedanib as a striking alternative to prevent fibrotic processes in the ophthalmic field. The ECM components fibronectin and collagen I we found to be similarly inhibited to the IPF-study from Knüppel et al. [13]. Moreover we found an almost complete reduction of α -SMA expression, which was impressively confirmed by immunocytochemical analysis. The reduction of α SMA expression was even stronger compared to the study of Stahnke et al. that evaluated the pharmaceutical Pirfenidone [14]. Thus, Nintedanib could be a potential agent that prevents postoperative undesired excessive scarring reactions or fibrosis in the eye through specific mechanisms of action with few side effects.

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