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Actinomycin D for fibrosis management in ophthalmic implant surgery

Abstract: Implants that feature a drug delivery system loaded with antifibrotic active drugs provide a promising approach to address postoperative complications caused by fibrosis. This study is intended to clarify whether Actinomycin D has an impact specifically on components of the extracellular matrix (ECM) and its formation in human primary fibroblasts of the Tenon capsule (hTF). Furthermore, the suitability of this agent in poly(N-vinylpyrrolidone)-poly(methylmethacrylate)(PVP-co-PMMA) as a drug delivery model is evaluated in drug incorporation and release studies.

RT-qPCR revealed a significant downregulation of the fibrotic marker genes *ACTA2*, *COL1A1* and *FNI* in cells stimulated with TGF- β 1 and additionally treated with Actinomycin D. However, these findings could only be confirmed on α -SMA protein level. collagen I and Fibronectin synthesis stayed unaffected. The diffusion based incorporation of Actinomycin D into the polymer model proved to be very effective. The release of the agent was retarded with a slightly prolonged kinetic. These findings make Actinomycin D a promising antifibrotic agent in ophthalmic implant surgery.

Keywords: fibrosis, ophthalmic implant, drug delivery, Actinomycin D

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1 Introduction

In ophthalmic implant surgery, fibrosis is still one of the main causes for postoperative complications. For instance, in trabeculectomy, a surgical procedure to reduce intraocular pressure, 47% of patients suffer from obstruction of the aqueous humor drainage pathway within the first 5 years [1]. Moreover, in about 30% of the patients with glaucoma drainage implants (GDI) postoperative fibrosis results in encapsulation and stenosis of the implant [2]. A further example for the occurrence of fibrosis in the ophthalmological field is the so called posterior capsular opacification (PCO) after implantation of artificial lenses [3]. Capsular fibrosis ceases in opacity of the posterior capsule and occurs in healthy adults with an incidence of 4 - 12% after standard cataract surgery without complications [4].

Actinomycin D has been used as an anti-tumor drug in clinical practice since 1954. It exerts its anti-tumour activity by inhibiting protein biosynthesis through an intervention in mRNA synthesis [5]. Inhibition occurs through the intercalation of Actinomycin D into the DNA helix [6] and also through prevention of the uncoiling of the DNA by inhibiting the topoisomerase I and II complexes [7].

Actinomycin D releasing implants may have an influence on excessive cellularisation of the implant surfaces due to its cytostatic property. Moreover, Actinomycin D might inhibit enhanced ECM formation of the physiological highly active fibroblasts.

ACTA2 (α -smooth muscle actin, α -SMA), *COL1* and *FNI* genes have a significant impact on the formation of the ECM [8]. The α -SMA protein is highly expressed in myofibroblasts, which secrete large amounts of ECM proteins during wound healing, and moreover it has a key role in contraction of healing wounds [9]. Collagens are a protein family secreted by fibroblasts as the basic component of ECM [10]. Fibronectins are cell surface glycoproteins interacting with diverse ECM proteins like collagen, fibrin and integrin [11]. It has recently been shown, that secretion of these ECM factors was strongly induced in TGF- β 1 stimulated hTFs [12].

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2 Materials and methods

2.1 Cell culture

Human primary fibroblasts from the Tenon capsule (hTF) 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 1 mg glucose, 1 % Penicillin/Streptomycin, 10 % fetal calf serum (FCS) and 3.7 g/L NaHCO₃ under standard cell culture conditions (37 °C, 5 % CO₂).

2.2 Actinomycin D

For all experiments Actinomycin D (~ 98 % purity, MERCK, Darmstadt, Germany) was dissolved in ethanol to a 1 mM stock solution. For cell culture experiments Actinomycin D stock solution was further diluted in DMEM and finally used in a concentration of 10 nM. In drug release studies the 1 mM stock solution was used for drug incorporation.

2.3 Fibrosis model

To analyse the antifibrotic potential of the cytostatic agent hTFs were seeded into eight cell culture flasks and incubated until 70 % confluence in DMEM with FCS. Before treatment, cells were incubated for 24 h in DMEM without FCS. After that starving medium was removed and DMEM containing the test substances was applied to the cells. There were four different treatment groups, DMEM with 10 nM Actinomycin D, with 10 ng/mL TGF-β1, with both 10 nM Actinomycin D and 10 ng/mL TGF-β1. Pure DMEM was used as control group. All treatments were performed in two cell culture flasks, one for mRNA and one for protein extraction. Experiments were carried out as triplicates with hTF from three different donors.

2.4 Real-time quantitative PCR (RT-qPCR)

For RNA preparation, cells were treated with TRIZOL reagent (Ambion). Further purification of RNA was

conducted with RNeasy mini kit (Qiagen). Synthesis of cDNA was realised with First strand cDNA synthesis kit and RT-qPCR was carried out with DyNamo HS SYBR Green qPCR kit (Thermo Scientific) in Eppendorf realplex² Mastercycler. Oligonucleotides (Table 1) were deduced with 20 to 22 bp and a melting temperature of 64 to 66 °C.

Table 1: Oligonucleotides used in RT-qPCR

gene	Sense (3'-5')	Antisense (3'-5')
<i>EEF1A1</i>	GGCTTCACTGCTCAGG TGATTA	GGCAGCATCACCAGAC TTCAA
<i>RPS18</i>	CGAAGATATGCTCATGT GGTGTT	GGCTAGGACCTGGCTG TATTT
<i>PPIA</i>	GTGGTGACTTCACACG CCATA	GCCATCCAACCACTCA GTCTT
<i>GAPDH</i>	GGTAAAGTGGATATTGT TGCCAT	GAGGGATCTCGCTCCT GGAA
<i>ACTA2</i>	AGGAGAAACTGTGTTAT GTAGCT	GACTCCATCCCAGTGA AGGAT
<i>COL1A1</i>	GGCTGCAACCTGGATG CCAT	CCATACTCGAACTGGA ATCCAT
<i>FN1</i>	GCCAGTGCTTAGGCTT TGGAA	CCTCATGAGGGTCACA CTTGAA

Ct values of the treated groups were first normalised with geometric mean of the housekeeping genes *EEF1A1*, *RPS18*, *PPIA* and *GAPDH* and subsequently with the untreated control group. Significances were determined with one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test.

2.5 Western Blot

For the SDS-PAGE, Laemmli sample buffer was added directly after washing to the cell culture flasks in order to denature the proteins immediately. The protein concentration was determined with the Pierce 660nm Protein Assay Kit and 10 µg to 30 µg were added to 7.5% or 10% polyacrylamide gels followed by protein transfer to PVDF membranes via Western Blot procedure. For protein detection, mouse monoclonal antibodies anti-α-SMA (Abcam, 1:500), anti-collagen I (Abcam, 1:500), anti-fibronectin (Sigma, 1:1000) and anti-β-tubulin as a reference were used. Primary antibodies were combined with the secondary antibody Goat Anti-Mouse IgG H&L (HRP) from Abcam.

2.6 Implant material and drug release

Drug incorporation and release studies were performed with poly(N-vinylpyrrolidone)-poly(methylmethacrylate)(PVP-co-PMMA) as a model drug delivery system. For drug incorporation three cylindric specimens were given into 1 mM Actinomycin D solution. One specimen was given into a volume of 100 mL Actinomycin D solution, one in 200 mL and one in 300 mL. Analysis of temporal drug release from the test specimens was performed *in vitro* in 2 mL of 0.9 % saline solution at 37°C. Specimens were not agitated. After a defined time of incubation, the saline solution was collected and drug content was analysed by means of high performance liquid chromatography (HPLC; KNAUER Wissenschaftliche Geräte GmbH, Berlin, Germany).

3 Results

3.1 Actinomycin D diminishes *ACTA2*, *COL1A1* and *Fn1* mRNA level

RT-qPCR analysis revealed a significant upregulation of *ACTA2*, *COL1A1* and *FN1* gene in TGF- β 1 treated hTFs as a proof for functionality of the fibrosis model (Figure 1).

Figure 1: Relative transcription ($\Delta\Delta C_t$) of *ACTA2*, *COL1A1* and *FN1*. Hash indicates significant upregulation in TGF- β 1 treated hTFs, asterisk indicate significant Actinomycin D effected suppression of the respective gene (*/# $p < 0.05$; **/### $p < 0.01$; ***/### $p < 0.001$).

The TGF- β 1 induced upregulation of these ECM factors was significantly and unmitigatedly blocked with 10 nM Actinomycin D included to the cell culture medium.

3.2 Actinomycin D reduces α -SMA

Western Blot could only partially confirm the findings on mRNA level. In TGF- β 1 stimulated hTFs protein level of α -SMA was clearly higher compared to the untreated control group (Figure 2).

Figure 2: Western Blot with anti- α -SMA antibody. Anti- β -Tubulin antibody was used as reference.

The supplementation of Actinomycin D blocked the excessive expression of α -SMA, while Tubulin stayed unaffected in all treatment groups. However, this attenuating effect of Actinomycin D could only be confirmed for α -SMA, but not for COL1A1 and FN1 proteins.

3.3 Drug release studies

Specimens were loaded with different amounts of Actinomycin D stock solution. Drug release was investigated for 19 hours (Figure 3).

Figure 3: Drug release kinetic (cumulative) of Actinomycin D in PVP-co-PMMA. Specimens stored in different amounts of drug stock solution for loading.

After two hours 70-80% of the total drug amount was released. Release was triggered by diffusion. Degradation of the copolymer during that time could not be observed. Between the specimens, no difference in the release profile normalised to the total drug load was found as it is expected for diffusion controlled systems.

4 Discussion

Increased expression of myofibroblast marker ACTA2 (α -SMA) has been reported for different pathologies in wound healing processes of the eye [14, 15]. The damping of ACTA2 expression, as well as the prevention of myofibroblast differentiation, could therefore be a possibility to attenuate such pathological processes. In comparison to our experiments with Actinomycin D, a similar effect was demonstrated in hTFs treated with Pirfenidone, a drug against idiopathic pulmonary fibrosis [12]. Moreover, compared to Pirfenidone Actinomycin D also decreased FN mRNA-concentration. On the other hand Pirfenidone had an impact on protein level of collagens, which was not found for Actinomycin D. To obtain a more comprehensive understanding of the antifibrotic potential of Actinomycin D, further ECM factors should be included in the analysis. Furthermore, a higher number of donors should be considered.

In addition to the evaluation of the antifibrotic potential of the active substance, the second task of this study was to determine whether Actinomycin D is generally suitable for the use in drug release systems. The diffusion-determined uptake of the active substance into the co-polymer PVP-co-PMMA proceeded very quickly and effectively. However, under physiological conditions (0.9% saline solution at 37°C), the active ingredient was for the most part released from the polymer just as rapidly. In this context, possibilities should be sought to chemically bind the active ingredient to suitable polymers and thus ensure a retarded and prolonged drug release, avoiding an initial burst release.

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