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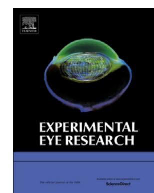

A TGF-β receptor 1 inhibitor for prevention of proliferative vitreoretinopathy

Khaled Nassar [a,b,*,1], Swaantje Grisanti [a,1], Aysegul Tura [a], Julia Lüke [a], Matthias Lüke [a], Mahmoud Soliman [c], Salvatore Grisanti [a]

[a] University of Luebeck, Department of Ophthalmology, Ratzeburger Allee 160, D-23538 Luebeck, Germany
[b] Fayoum University, Department of Ophthalmology, 63514 Fayoum, Egypt
[c] Cairo University, Department of Ophthalmology, 11956 Cairo, Egypt

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ABSTRACT

This study evaluates the use of the TGF-β receptor 1 inhibitor LY-364947 (LY) to prevent proliferative vitreoretinopathy (PVR). For the in vitro experiments Human Tenon's Fibroblasts (HTFs) and retinal pigment epithelial (RPE) cells were treated with different concentrations of LY to determine HTF proliferation and RPE transdifferentiation. For in vivo testing 30 rabbits underwent a PVR trauma model. The animals received different concentrations of intravitreally injected LY, with or without vitrectomy. LY treatment reduced HTF proliferation and RPE transdifferentiation in vitro. In vivo intravitreal injection of LY prevented PVR development significantly. This positive effect was also present when LY injection was combined with vitrectomy. Intravitreal injection of LY prevented tractional retinal detachment in 14 out of 15 animals. In conclusion, treatment with the TGF-β receptor 1 inhibitor LY reduces HTF proliferation and RPE transdifferentiation in vitro and prevents proliferative vitreoretinopathy and subsequent tractional retinal detachment in vivo.

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1. Introduction

Proliferative vitreoretinopathy (PVR) is a complex process involving cell proliferation of a variety of cells, secretion of pro-proliferative factors as well as remodelling processes of the extracellular matrix (ECM) (Ryan, 1985). Retinal glial cells (Mueller cells and astrocytes), retinal pigment epithelial (RPE) cells, macrophages and hyalocytes are the origin of PVR membranes (Hirayama et al., 2004; Laqua and Machemer, 1975; McLeod et al., 1987). Epithelial–mesenchymal transition (EMT) is a process in which epithelial cells lose their differentiated phenotypes and acquire mesenchymal characteristics. During retinal detachment RPE cells become dislodged from their monolayer and move into the vitreous cavity or subretinal space. They adhere to the detached retina, proliferate and undergo EMT to gain a fibrotic phenotype (Tamiya et al., 2010). Transdifferentiation of RPE cells is accompanied by a shift in their biologic activities and goes along with an enhancement of their contractile potentials (Grisanti and Guidry, 1995).

While surgical techniques in vitrectomy for patients with PVR and anatomical outcome have improved over the time, functional success is still limited. Current research projects try to find a way to address the underlying disease at a cellular level, in order to reduce the need for surgical re-interventions and improve the functional outcome for the patient. Therefore, recent efforts have focused on potential adjuncts to surgical treatment of PVR. To date, only a few therapeutic agents have been tested in human clinical trials: corticosteroids (Ahmadieh et al., 2008; Furino et al., 2003; Jonas et al., 2000; Yamakiri et al., 2008); retinoic acid (Araiz et al., 1993); 5-Fluorouracil (5-FU) (Blumenkranz et al., 1984), daunorubicin (Wiedemann et al., 1991) and combinations of heparin with 5-FU (Sundaram et al., 2013), heparin with dexamethason (Williams et al., 1996) or heparin with retinoic acid (Chang et al., 2008). However, no

Abbreviations: PVR, Proliferative Vitreoretinopathy; TRD, Tractional Retinal Detachment; TGF-β, Transforming Growth Factor beta; TGF-β receptor 1, Transforming Growth Factor beta receptor 1; LY, LY-364749; ECM, Extracellullar Matrix; HTF, Human Tenon's Fibroblasts; RPE, Retinal Pigment Epithelial, Retinal Pigment Epithelium.

* Corresponding author. University of Luebeck, Department of Ophthalmology, Ratzeburger Allee 160, D-23538 Luebeck, Germany. Tel.: +49 451 500 4010/3090; fax: +49 451 500 4952.

E-mail addresses: khaledmohnassar@yahoo.com, khalnassar@gmail.com (K. Nassar).

[1] K. Nassar and S. Grisanti are equivalent First Authors.

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satisfactory results were achieved so far. Therefore the need for more experimental work to find an effective and safe therapeutic agent is still present.

TGF-β is a multifunctional cytokine, regulating pivotal biological responses, such as differentiation, apoptosis, migration, immune cell function, and ECM synthesis (Massagué, 1998). TGF-β has three isoforms (TGF-β1–3) and is secreted in a biologically inactive form. Latent TGF-β is activated by various chemical or enzymatic treatments (Brown et al., 1990). It has been implicated in tissue contraction in fibrous diseases, such as liver cirrhosis, pulmonary fibrosis, and systemic sclerosis (Border and Noble, 1994).

In the eye, TGF-β is over-expressed in the vitreous of patients with proliferative diabetic retinopathy (PDR) and PVR (Kita et al., 2007). TGF-β is presumed to contribute to the contraction of cicatricial membranes and subretinal strands in PVR (Winkler and Hoerauf, 2011). Several inhibitors that interfere with the TGF-β pathway were tested as anti PVR modalities, including fasudil (Itoh et al., 2007), simvastatin (Kawahara et al., 2008), troglitazone (Cheng et al., 2008), glucosamine (Liang et al., 2011), decorin (Nassar et al., 2011) and wortmannin (Yokoyama et al., 2012).

The current study evaluates the antiproliferative effect of the TGF-β receptor 1 inhibitor LY-364947 on tissues of the eye, using an in vitro experimental setting and an in vivo model (Cleary and Ryan, 1979a, 1979b; Nassar et al., 2011). LY-364947 is a selective pyrazole-based inhibitor of the TGF-β receptor 1 kinase domain. It less effectively inhibits TGF-β receptor 2, p38 MAPK and mixed lineage kinase-7 (Sawyer et al., 2003). Vogt et al. reported that LY-364947 was able to inhibit TGF-β signalling with potential off target effects (Vogt et al., 2011). Other reports suggest that the LY-364947 effects are independent from the cell type (Fretz et al., 2008; Singh et al., 2012; Vogt et al., 2011). An anti-fibrotic effect of this drug was previously reported in silicosis treatment (Xu et al., 2012), cancer research (Gauger et al., 2011) and following central nervous system injury (Kimura-Kuroda et al., 2010).

2. Materials and methods

2.1. In vitro experiments

2.1.1. Fibroblast cell culture

Human Tenon's Fibroblasts (HTF) were isolated as described previously (Tura et al., 2007) and grown in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% heat-inactivated foetal calf serum ("culture medium", Invitrogen-Gibco Life Technologies, Karlsruhe, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin (Biochrom, Berlin, Germany). Cells from passages 3 to 9 were used in all experiments as suggested before (Meske et al., 2005).

2.1.2. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) test (HTF)

HTF were seeded at 5×10^3 cells/well ($n = 6$) in 96 well plates, grown initially for 36 h and incubated with LY-364947 (Sigma–Aldrich, Munich, Germany) diluted at the final concentrations of 0, 5, 20, 50, and 100 μmol (μM) in culture medium for 30 min. An MTT test was done as previously described (Barile, 2007) and the absorbance at 570 nm was measured using a microplate reader (Tecan Group Ltd., Maennedorf, Switzerland). The standard error of mean (SEM) of 3 independent tests was calculated. To estimate the IC50, a 4 parameter logistic nonlinear regression model was used (GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego California USA).

2.1.3. Cell proliferation (HTF)

HTF from passage 5 at a concentration of 5.0×10^3 were plated onto sterile 16-well Lab-Tek Chamber slides (Thermo scientific, USA) and allowed to reach 50% confluence for 48 h. Then the cells were incubated with the test substance for 48 h. 10 μM BrdU (5-Bromo-2′-deoxy-uridine Labelling and Detection Kit I, Roche, Mannheim, Germany) was added for 60 min at 37 °C, 5% CO_2. Detection of BrdU incorporation was performed following the manufacturer's instructions and analysed by fluorescence microscopy (Leica DMI 6000 B microscope, Wetzlar, Germany).

2.1.4. Culture of retinal pigment epithelium (RPE)

A non polarized RPE cell culture was used to evaluate the effects of LY-364947. Bovine eyes were isolated as described (Edwards, 1981; Yanagihara et al., 1996) and cultured in culture medium supplemented with 1% sodium pyruvate and 10% FBS (High Serum Medium = HSM) at 37 °C under 5% CO_2. Upon reaching confluence, half of the medium in each well was discarded and replaced by low serum medium (LSM) containing 2% FBS. Then the cells were allowed to differentiate in LSM for 4–5 weeks, with medium change every 2–3 days.

2.1.5. Incubation of RPE with LY-364947

RPE cells (passage 1–3) were trypsinized for 10 min, collected in RPE-medium, centrifuged at 300 g for 8 min, resuspended in fresh HSM-medium, seeded into 24-well fluorocarbon plates (Zell-Kontakt, Noerten-Hardenberg, Germany) at a concentration of 1×10^4 cells/well, and grown until reaching confluence (5–7 days). Afterwards, culture medium was replaced by HSM plus LY-364947 or by LSM containing TGF-β plus LY-364947. Different concentrations of LY-364947 were used: 0, 1, 5, 20 or 50 μM. These concentrations were chosen according to previous in vitro studies (Kano et al., 2007; Sethi et al., 2011; Shiou et al., 2006; Vogt et al., 2011). Cells were incubated further for 4 weeks with medium change every 2–3 days and processed for immunofluorescence staining.

2.1.6. Immunofluorescence staining of RPE cells

Cells were fixed in 2% paraformaldehyde (PFA) followed by 4% PFA for 10 min respectively. Immunostaining was performed as described (Tura et al., 2007), using primary antibodies against β-catenin (1:1000 dilution in blocking buffer, Abcam, Cambridge, UK) or alpha smooth muscle actin (α-SMA) (1:50 dilution, Abcam), followed by Cy3- or Alexa 488-conjugated anti-rabbit antibodies (diluted 1:200 and 1:100 in blocking buffer; Jackson Immuno-Research, Hamburg, Germany; Molecular Probes, Darmstadt, Germany, respectively). Double staining of α-SMA with actin filaments was performed as described above, using TRITC conjugated secondary antibodies (1:50, Sigma Aldrich) followed by the incubation in Alexa Fluor 488-phalloidin to stain the actin filaments (1:50 in blocking buffer, Molecular Probes) for 30 min. Nuclei were counterstained with DAPI (1 μg/ml in PBS) for 10 min. The wells were excised using a scalpel and mounted in Mowiol (Sigma–Aldrich, Munich, Germany).

2.1.7. Quantification

The results of the BrdU incorporation and the RPE trans-differentiation model were analysed by fluorescence microscopy. Immunopositive cells were counted by a semi automated method

Table 1
In vivo study groups.

Group	Description
G1	Traumatic PVR only ($n = 5$).
G2	Traumatic PVR + intravitreal injection of 20 μM/0.1 ml LY-364947 ($n = 5$).
G3	Traumatic PVR + vitrectomy ($n = 5$).
G4	Traumatic PVR + vitrectomy + vehicle (DMSO) "sham" ($n = 5$).
G5	Traumatic PVR + vitrectomy + 5 μM/0.1 ml LY-364947 ($n = 5$).
G6	Traumatic PVR + vitrectomy + 20 μM/0.1 ml LY-364947 ($n = 5$).

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