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Original research article

Transforming growth factor β-related genes in human retinal pigment epithelial cells after tacrolimus treatment

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ABSTRACT

Background: The transforming growth factor β (TGFβ) family plays an important role in the pathogenesis of many diseases, including fibrotic pathologies of the eyes. The difficulties of surgical procedures contribute to the search for new treatment strategies for proliferative vitreoretinopathy. Therefore, the aim of this study was to investigate the expression profile of TGFβ isoforms, their receptors, and TGFβ-related genes in human retinal pigment epithelial cells (RPE) after tacrolimus (FK-506) treatment in the presence or absence of lipopolysaccharide (LPS)-induced inflammation.
Methods: The expression profile was analyzed using oligonucleotide microarrays and quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) techniques.
Results: Analysis using oligonucleotide microarrays revealed 20 statistically significant differentially expressed TGFβ-related genes after LPS treatment in relation to control cells, and after tacrolimus and LPS treatment in relation to LPS-treated cells. Moreover, our results showed that mRNA levels for *TGFβ2* and *TGFβR3* after tacrolimus treatment, and for T*GFβR3* after tacrolimus and LPS treatment in RPE cells were decreased. In turn, in the presence of LPS-induced inflammation, *TGFβ2* mRNA level was increased.
Conclusions: These results can be important in regard to the treatment of proliferative vitreoretinopathy, pathogenesis of which is associated with processes regulated by TGFβ, such as inflammation, proliferation, epithelial–mesenchymal transition (EMT), and fibrosis.

Introduction

The transforming growth factor β (TGFβ) family includes over 40 members. The most important subfamilies seem to be TGFβ, bone morphogenetic protein (BMP), growth and differentiation factor (GDF), Müllerian inhibitory factor (MIF), activin, and inhibin [1]. In mammals, the TGFβ subfamily consists of three isoforms (TGFβ1, TGFβ2, and TGFβ3) [2]. TGFβs are involved in regulation of various processes such as cell growth, differentiation, proliferation, adhesion, migration, inflammation, and wound healing [1]. Additionally, TGFβ isoforms can play an important role in the pathogenesis of many human diseases, including fibrotic pathologies of the eyes [1,3].

Proliferative vitreoretinopathy (PVR), a blinding disorder, is characterized by increased proliferation of retinal pigment epithelial cells (RPE), glial cells, fibroblasts, and inflammatory cells [4]. Proliferation of all the above-mentioned cells can consequently lead to the formation of fibrotic, contractile membranes on the inner and outer retinal surfaces. Moreover, RPE cells within diseased eyes can lose their epithelial morphology and undergo epithelial–mesenchymal transition (EMT) [5]. PVR appears to be a tissue repair process induced by retinal damage or inflammation. Previous research has revealed that pro-inflammatory cytokines such as interleukin 1β (IL1β), IL2, IL6, and IL8 and growth factors such as TGFβ and platelet-derived growth factor (PDGF) can play a major role in the development of this disease [5,6]. Currently, surgery is the standard treatment for PVR. However, the difficulties of the surgical procedure contribute to the search for new treatment strategies for PVR [4].

Tacrolimus (FK-506) is an immunosuppressive and anti-inflammatory drug [7]. In ophthalmology, tacrolimus is effective

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in the treatment of uveoretinitis, allergic conjunctivitis, and corneal graft rejection [8–10]. However, there are only a few published reports regarding the use of tacrolimus in the treatment of ocular diseases related to dysfunctions of RPE cells, including PVR [7,11]. Moreover, the effect of tacrolimus on the regulation of TGFβ signaling pathways is still not fully understood. Therefore, the aim of this study was to investigate the expression profile of TGFβ isoforms, their receptors, and TGFβ-related genes in human RPE cells after tacrolimus treatment in the presence or absence of lipopolysaccharide (LPS)-induced inflammation using oligonucleotide microarrays and quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) techniques.

Materials and methods

Cell culture conditions

Human retinal pigment epithelial cells (H-RPE cell line) were obtained from Clonetics (194987, Clonetics, San Diego, CA, USA) and routinely maintained in retinal pigment epithelial basal medium (RtEBM™ Medium, Lonza, Basel, Switzerland), supplemented with human fibroblast growth factor basic (FGFB), L-glutamine, and gentamicin (RtEGM™ SingleQuots™, Lonza, Basel, Switzerland) at 37 °C in a 5% CO_2 incubator (Direct Heat CO_2 Incubator, Thermo Scientific, Waltham, MA, USA). RPE cells were used from passages 4 to 6 in the experiments.

Cytotoxicity test

Cytotoxicity of lipopolysaccharide and tacrolimus against RPE cells was tested using a sulforhodamine B-based *in vitro* toxicology assay kit (TOX6, Sigma–Aldrich, St. Louis, MO, USA) and a Wallac 1420 VICTOR plate reader (Perkin Elmer, Waltham, MA, USA), according to the manufacturer's instructions.

The RPE cells were seeded into 96-well culture plates (Nunc GmbH, Wiesbaden, Germany) at a density of 6×10^3 cells/well. The RPE cells were then treated with 1, 2, and 10 μg/mL lipopolysaccharide (LPS *Escherichia coli* 055:B5, L2880; Sigma–Aldrich, St. Louis, MO, USA) and 0.01, 0.1, 1, 10, 100, 1000, and 2500 ng/mL tacrolimus (FK-506 monohydrate, F4679; Sigma–Aldrich, St. Louis, MO, USA) for 24 h to measure possible cytotoxicity.

Treatment of RPE cells

The concentrations of LPS and FK-506 were chosen experimentally (data not shown) and on the basis of available literature data [12–14]. RPE cells were seeded into 6-well culture plates (Nunc GmbH, Wiesbaden, Germany) at a density of 1×10^4 cells/cm^2. After reaching confluence, cells were treated with 1 μg/mL lipopolysaccharide and 10 ng/mL tacrolimus for 6 h. Tacrolimus was added to the cultures 30 min prior to the LPS treatment. In separate cultures, cells were incubated with LPS or tacrolimus alone at the indicated concentrations and for the indicated time. Control cells were left untreated.

The cell samples were collected in triplicate, washed with phosphate buffered saline (PBS), and stored at −70 °C for 48 h until further molecular analysis.

Ribonucleic acid extraction

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA extracts were treated with DNase I (RNeasy Mini Kit, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quality of extracts was checked electrophoretically using 0.9% agarose gel stained with ethidium bromide (Sigma–Aldrich, St. Louis, MO, USA). The results were analyzed and recorded using the 1D Bas-Sys gel documentation system (Biotech-Fischer, Perth, Australia). Nucleic acids concentration was determined using a GeneQuant II RNA/DNA spectrophotometer (Pharmacia Biotech, Cambridge, UK).

Oligonucleotide microarray analysis

Analysis of the expression profile of TGFβ-related genes was performed using commercially available oligonucleotide microarrays of HG-U133A 2.0 (Affymetrix, Santa Clara, CA, USA) in accordance with the manufacturer's recommendations, as described previously [15].

The oligonucleotide microarray analysis was performed on twelve samples: three samples of untreated control RPE cells (C), three samples of RPE cells treated with FK-506 (FK-506), three samples of RPE cells treated with LPS (LPS), and three samples of RPE cells treated with FK-506 and LPS (FK-506/LPS).

Quantitative real-time reverse transcription polymerase chain reaction assay

Detection of the expression of *TGFβ1*, *TGFβ2*, *TGFβ3*, *TGFβR1*, *TGFβR2*, *TGFβR3*, and β-actin (*ACTB*) mRNAs was carried out using real-time RT-qPCR with a Quantitect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) and an Opticon™ DNA Engine Continuous Fluorescence detector (MJ Research) as described previously [15]. All samples were tested in triplicate. Oligonucleotide primers specific for *TGFβ1*, *TGFβ2*, *TGFβ3*, *TGFβR1*, *TGFβR2*, *TGFβR3*, and *ACTB* were described by Strzalka et al. and Jachec et al. [16,17].

Statistical analyses

Microarray data analysis was performed using the GeneSpring 13.0 platform (Agilent Technologies UK Ltd, South Queensferry, UK). TGFβ-related genes (1101 transcripts for 677 genes) were selected from the NetAffx Analysis Center database of Affymetrix (http://www.affymetrix.com/analysis/index.affx). The normalized microarray data were used to compile a list of selected genes whose expression appeared to be up- or down-regulated by a cutoff of at least 2-fold change. *T* test unpaired was applied to detect differentially expressed genes at $p < 0.05$.

Values obtained by real-time RT-qPCR and cytotoxicity data were expressed as means and standard deviation (SD). Statistical analyses were performed using Statistica 9.0 software (StatSoft, Tulsa, OK) and the level of significance was set at $p < 0.05$. The one-way ANOVA and Tukey *post hoc* tests were applied to compare the cytotoxicity data. *T* test unpaired was applied to evaluate differences in the expression of examined genes between untreated cells and cells treated with FK-506 and LPS.

Results

Cytotoxicity measurement

The cytotoxicity assay of the LPS effect (1, 2, and 10 μg/mL) on RPE cell viability showed no statistically significant differences compared to the controls, confirming that LPS is not cytotoxic at the tested concentrations for RPE cells (ANOVA, $p = 0.4379$). There was statistically significant change in the viability of RPE cells after exposure to tacrolimus concentrations of 0.01, 0.1, 1, 10, 100, 1000, and 2500 ng/mL (ANOVA, $p = 0.0016$) (Fig. 1). Cells exposed to FK-506 at a concentration of 1000 ng/mL showed significant decrease in cell viability compared to cells exposed to tacrolimus concentrations of 0.1 ng/mL (Tukey *post hoc*, $p = 0.0260$), 1 ng/mL (Tukey *post hoc*, $p = 0.0473$), and 10 ng/mL (Tukey *post hoc*,