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Overexpression of low-density lipoprotein receptors stimulated by vascular endothelial growth factor in fibroblasts from pterygium

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ABSTRACT

The activation of subconjunctival fibroblasts is believed to be responsible for the pathogenesis of pterygium. Vascular endothelial growth factor (VEGF) appears to be the most potent stimulator of formation and progression of pterygium. Pterygium excision is a common procedure, although the recurrence rates remain high. Various postoperative adjuvant therapies are now attempted to lower the recurrence rate, with severe side effects. To offer a greater therapeutic effect and lower side effects, it's necessary to discover a constant nanoparticle drug delivery targeting to subconjunctival fibroblasts in pterygium (PSFs). This study was designed to investigate the expression of low-density lipoprotein receptor (LDLr) stimulated by VEGF in PSFs. We found that after exposure to VEGF, mRNA and protein levels of LDLr were both increased significantly in PSFs, assessed using relative quantitative real-time RT-PCR and Western blot. Moreover, it's demonstrated that the expression of LDLr were positively correlated with the cells proliferation. Uptake of DiI–LDL via live PSFs was increased with time, estimated by confocal microscopy. The protein expression of LDLr in pterygium subconjunctival tissues was significantly higher than in normal subconjunctival tissues. These results suggest that LDLr in the activated PSFs may become a novel target receptor for controlled drug delivery to lower postsurgical recurrence rate.

1. Introduction

Pterygium is a disease associated with proliferation of fibrovascular tissues underneath the conjunctiva into the cornea, causing ocular irritation, astigmatism, and visual disturbance, which is related to many factors including chronic stimulation, climate, inflammation and genetics [1]. It has been estimated that in some counties, about 22% of the general population have pterygium that may necessitate surgical removal [2]. Currently, conjunctival auto-transplantation is a common procedure for pterygium, with recurrence rates ranging from 2% to 39% [3,4]. Among various mediators involved either directly or indirectly in the pathogenesis of pterygium, such as VEGF, basic fibroblast growth factor (bFGF), interleukin-1 (IL-1), IL-6, IL-8, epidermal growth factor (EGF), heparin-binding EGF (HB-EGF), tumor necrosis factor (TNF) and transforming growth factor (TGF), VEGF appears to be the most potent stimulator for the formation and progression of fibrovascular tissues [5,6]. Several studies have also showed that VEGF can stimulate the proliferation of Tenon's capsule fibroblasts *in vitro* [7,8].

Furthermore, the activation of subconjunctival fibroblasts is believed to be responsible for the subconjunctival fibrovascular proliferation that characterize pterygium [1,9]. In our previous studies, we have found that LDLr is upregulated in activated human subconjunctival fibroblasts (HSFs) by TGF-β_2[10] and the overexpressed LDLr in the activated HSFs collected the photosensitizer mainly inside to arouse phototoxicity [11]. LDLr is a single-chain transmembrane glycoprotein that specifically mediates binding and endocytosis of plasma LDL. As LDL plays an important role for the transport and release of cholesterol in the blood to cells, overexpression of LDLr is attributed to supply cholesterol for membrane synthesis associated with cellular proliferation. Therefore, it has been an interest that LDL may serve as a target candidate in drug-delivery [12]. Recent studies have shown that

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constant targeted drug nanoparticle delivery may offer a more effective therapy than traditional dosing methods [13].

We assumed that the addition of VEGF might increase the activity of fibrovascular proliferation in pterygium. The present study was conducted to investigate the status of LDLr in PSFs after stimulated by VEGF *in vitro* as well as it's expression in pterygium subconjunctival tissues.

2. Materials and methods

2.1. Specimen collection and culture of fibroblasts

Pterygium subconjunctival tissues specimens were obtained from the patients with primary pterygium at the central portion of the pterygium body during excision procedure (n = 10; 4 males, 6 females; ages: 56.1 ± 3.96 years; PSFs). The normal subconjunctival biopsies were collected from the volunteers who had age-related cataract at the time of cataract surgery with superior scleral tunnel incision (n = 9; 5 males, 4 females; ages: 54.3 ± 2.53 years; HSFs). Each tissue specimen was divided into two parts. One part for cell culture, and the rest for quantification analysis of LDLr protein. The research procedures were performed in compliance with the Declaration of Helsinki, and ethics approval was obtained from Ethics Committee of Eye and ENT Hospital of Fudan University. The informed written consent was obtained from all subjects. Patients with other ocular diseases/ocular surgery, or with systemic diseases were excluded. The nine HSFs donors performed cataract surgery without the diagnosis of pterygium. The fibroblasts obtained were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum for 48 h. Then they were starved in serum free DMEM (SFDMEM) for 24 h. Thereafter, recombinant VEGF (R&D Systems, USA & Canada) was added to the serum free DMEM media for 1, 3 and 5 d. Final concentrations of VEGF were 0 (the control), 200, 400, 600, 800, and 1000 pg/mL [14], respectively.

2.2. Immunofluorescence staining of cultured fibroblasts

Fibroblasts (PSFs and HSFs) ($1.0–2.0 \times 10^5$ cells/coverslip) were plated onto sterile coverslips. Then they were stained with anti-Vimentin and anti-Fibronectin (all 1:200 dilution; Sigma-Aldrich) antibodies. Immunofluorescence staining was performed as described previously [10]. The samples were viewed and photographed using a confocal microscope (510 UV–vis Meta, Zeiss).

2.3. MTS assay

PSFs and HSFs were plated in 96 well plates (Falcon) at the density of 2000 cells/well, and stimulated with various concentrations of VEGF. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H −tetrazolium (MTS) assay was performed by adding 20 μL of CellTiter 96 Aqueous One Solution Reagent (Promega Corporation) per well with 100 μL serum-free DMEM and then incubated at 37 °C in humidified 95% air/5% CO2 for 4 h. The absorbance was detected in multi-well spectrophotometer (Titertek Multiscan, Flow Lab, Scotland, UK) at 490 nm. Well containing basic medium served as controls.

Cell proliferation rate = (Treatment absorbance-Control absorbance)*100% / Control absorbance

2.4. Relative quantitative real-time RT-PCR

Fibroblasts were plated in 60-mm^2 dishes (Falcon, Oxford, UK) at a density of 25,000 cells/dish and stimulated by all concentrations of VEGF. The Trizol RNA reagent kit (Invitrogen Corp.) was used to extract the total RNA from the fibroblasts according to the manufacturer's instructions. Complementary DNA (cDNA) was acquired with a SYBR PrimeScriptTM RT-PCR Kit (TaKaRa Corp.). Relative quantitative real-time RT-PCR assay was conducted with ABI PRISM 7000 Sequence Detection System (TaKaRa Corp.). The relative quantity of LDLr mRNA were expressed as fold change = $2^{-\Delta\Delta Ct}$, normalized to GAPDH. The human LDLr primers and GAPDH oligonucleotide primers were used in the experiment as follow: LDLr-R: 5′-AGT CAC AGA CGA ACT GCC GAGA-3′; LDLr-F: 5′-CAA CGG CTC AGA CGA GCA AG-3′; GAPDH oligonucleotide primers: GAPDH-R: 5′-TGG TGA AGA CGC CAG TGG A-3′; GAPDH-F: 5′-GCA CCG TCA AGG CTG AGA AC-3′.

2.5. Western blot

The whole cellular proteins were isolated from cultured PSFs and HSFs. Total cell lysates were procured with lysis buffer containing 0.1 mM leupeptin, 1.6% Triton X-100, 1.5 mM phenylmethylsulfonyl fluoride, and 5 M urea. After centrifugation at 12,000 × *g* for 30 min, the supernatant was removed and stored at −80 °C. On the other hand, subconjunctival tissue specimens from primary pterygium and normal donor were sonicated in lysis buffer (Cell Signaling Technology, USA). Then they were centrifuged at 12,000 × *g* for about 20 min at 4 °C and stored at −80 °C. Protein concentrations were detected with the Bio-Rad protein assay. Equal amounts of 20 μg protein were resolved by SDS-PAGE. Then the proteins were transferred to a PVDF membrane, blocked in TBST at room temperature for 1.5 h later, then incubated with primary antibody (1:5000 for a monoclonal rabbit anti-LDLr from Abcam, UK) at room temperature for 2 h and with horseradish peroxidase-conjugated antibody against rabbit (1:1, 000 dilution; Cell Signaling Technology) for an additional 1.5 h. The bands were visualized using the ECL detection kit (Amersham International plc). An antibody specific to β-actin (1:1000 dilution; Cell Signaling technology) was used to strip and re-blot the same membrane. Signals were quantified with the Image Quant Image Analysis Software (Amersham Biosciences), which were normalized by β-actin levels.

2.6. Preparation of dil-LDL

Purified LDL was isolated from fresh plasma of healthy volunteers with gradient ultracentrifugation. Briefly, LDL was prepared from plasma layered with 1.063 g/ml KBr solution, spinning at 105,000 × *g* for 24 h. Filter-sterilized through a 0.22 μm filter, the LDL was dialyzed against 0.01% EDTA (ethylenediaminetetraacetic acid) and 0.9% saline. Prior to the following experiment, the purified LDL was labeled with DiI (1,1′-dioctadecyl- 3, 3, 3′, 3′-tetramethyl-indocarbocyanine perchlorate, Sigma), and evaluated by Lowry protein assay kit (Sigma, USA).

2.7. DiI-LDL uptake assay

PSFs and HSFs were plated in 30-mm^2 dishes at a density of 2.0×10^4 cells/dish and stimulated with the optimal final concentration and incubation time (according to the results of the MTS assay) of VEGF in DMEM supplemented with 0.1% FBS. The fibroblasts were treated with DiI-LDL (10 μg/mL) in DMEM at 37 °C in the incubator. Uptake via the live fibroblasts was photographed at × 40 magnification by Zeiss 510 UV–vis Meta microscopy at 1, 3, 6, and 9 h. Normalization was performed with basic medium. DiI fluorescence intensity in PSFs and HSFs were also quantified.

2.8. Statistical analysis

All data were analyzed by the analysis of variance (ANOVA), followed by Bonferroni analysis and student's *t* test analysis. The