

# Interleukin-6 receptor blockade suppresses subretinal fibrosis in a mouse model

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Received: 2013-12-20 Accepted: 2014-01-29

## Abstract

• **AIM:** To determine the involvement of the interleukin (IL) -6 with the development of experimental subretinal fibrosis in a mouse model.

• **METHODS:** Subretinal fibrosis was induced by subretinal injection of macrophage -rich peritoneal exudate cells and the local expression of IL -6 was assessed by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) at various time points. In addition, we investigated the effect of IL-6 receptor (IL-6R) monoclonal antibody (MR16-1) on subretinal fibrosis development.

• **RESULTS:** IL-6 mRNA level was significantly elevated at 1d after subretinal fibrosis induction and increased further to about 12-fold at 2d, reaching the peak. The result of ELISA showed that IL -6 protein was not detected in naive mice. At 2d after subretinal fibrosis induction, IL-6 protein level was upregulated to  $67.33 \pm 14.96$  pg/mg in subretinal fibrosis mice. MR16 -1 treatment resulted in a reduced subretinal fibrosis area by 48% compared to animals from control group at 7d.

• **CONCLUSION:** Our results indicated that IL-6 signaling may contribute to the pathogenesis of subretinal fibrogenesis and IL -6R inhibition may provide an effective, novel treatment of advanced and late -stage neovascular age-related macular degeneration.

• **KEYWORDS:** interleukin-6; subretinal fibrosis; age-related macular degeneration

**DOI:10.3980/j.issn.2222-3959.2014.02.02**

Cui W, Zhang H, Liu ZL. Interleukin-6 receptor blockade suppresses subretinal fibrosis in a mouse model. *Int J Ophthalmol* 2014;7 (2): 194-197

## INTRODUCTION

Neovascular age-related macular degeneration (AMD) leads to severe deterioration of central vision in elderly

individuals as the result of the development of choroidal neovascularization (CNV) in the macular region [1]. These new abnormal blood vessels first proliferate under the Bruch membrane and retinal pigment epithelium (RPE) and then invade the subretinal space, leading to subretinal hemorrhages, exudative lesions, serous retinal detachment, and disciform scars ultimately [2]. Local destruction of photoreceptors, RPE, and choroidal blood vessels leads to permanent reduction in macular function and vision.

Although molecular and cellular mechanisms underlying CNV are not fully elucidated, CNV is considered as a submacular wound healing process, requiring a continually evolving interaction among cells, cytokines and the extracellular matrix (ECM) [2,3]. Angiogenesis is an essential component of this process and current clinical strategies for treating CNV are primarily aimed at inhibiting vascular endothelial growth factor (VEGF), the major promoter of angiogenesis [4,5]. However, overall only 30% to 40% of neovascular AMD patients gain three lines in visual acuity, and roughly every sixth patient continues losing visual acuity and progresses to legal blindness even under standard treatment with potent VEGF inhibitors [6-8]. Moreover, a recent study documented the development or progression of submacular fibrosis after anti-VEGF therapy in patients with neovascular AMD [9]. The greater fibrotic responses after anti-VEGF therapy are thought to be due to an imbalance between complex interaction of angiogenesis and tissue fibrosis during wound healing process. It therefore raises the prospect that CNV may be amenable to therapies other than just anti-angiogenesis approaches. Ultimately, it is the scarring response that irreversibly damages photoreceptors, so therapies that modify this response may help preserve or even rescue photoreceptors.

Interleukin (IL)-6 is a pleiotropic cytokine that is involved in the acute phase of the inflammatory reaction, wound healing, angiogenesis and fibrogenesis [10]. Recent evidence has suggested that IL-6 may play important roles in the pathogenesis of ocular diseases. Vitreous aspirates from patients with proliferative diabetic retinopathy (PDR), another ocular wound healing process and vision-threatening disease characterized by retinal neovascularization, exhibit the parallel increases in IL-6 and VEGF [11]. In neovascular AMD, levels of IL-6 are proven to be related with the activity of CNV [12]. In a laser-induced CNV model, blockade of IL-6 signaling led to significant suppression of CNV [13]. However, no data have been reported to show the direct evidence of the pathogenic role of IL-6 in subretinal fibrosis,

observed in advanced and late-stage neovascular AMD. In this report, we show, for the first time, that local expression of IL-6 in the subretinal space is up-regulated during subretinal fibrosis development. Administration of IL-6 receptor (IL-6R) monoclonal antibody (MR16-1) potently attenuated subretinal fibrosis size. Thus, IL-6R inhibition may provide an effective, novel treatment of advanced and late-stage neovascular AMD.

## SUBJECTS AND METHODS

**Subjects** Female 7- to 10-week-old C57BL/6 (B6) mice were used in all experiments. Animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Methods

#### Induction and evaluation of subretinal fibrosis

Subretinal fibrosis was induced by subretinal injection of macrophage-rich peritoneal exudate cells (PECs), as previously described [14]. Briefly, B6 mice received an intraperitoneal injection of 2.5 mL of thioglycolate and the PECs were isolated three days later. A concentration of PECs of  $4 \times 10^7/\text{mL}$  was prepared for subretinal injection. Laser photocoagulation (0.1s, 200 mW, 532 nm diode laser, Iridex, Mountain View, CA, USA) was performed in the posterior pole of the retina of B6 mice, and 0.5  $\mu\text{L}$  of prepared PECs were injected into the subretinal space with a 10- $\mu\text{L}$  syringe (Hamilton Company, Reno, NV, USA) with a blunt-tipped needle. Eyes in which there was bleeding or no focal retinal detachment were excluded.

Seven days later, the area of glial fibrillary acidic protein (GFAP) was measured to quantify the subretinal fibrosis on choroidal flat mounts, as GFAP has been suggested to detect and quantitate subretinal fibrosis effectively in this animal model [14]. For GFAP staining, polyclonal rabbit anti-GFAP antibody (1:400, Dako, Glostrup, Denmark) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) were used. The flat mounts were observed with a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan) and the area was measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Quantitative real-time reverse transcription-polymerase chain reaction** Total RNA was extracted using Trizol reagent (Invitrogen), from RPE-choroid complexes of B6 mice on 1d, 2d, 3d, 5d and 7d after induction of subretinal fibrosis. Aliquots containing 1  $\mu\text{g}$  total RNA were reverse transcribed with an quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) kit (First-Strand cDNA Synthesis Kit; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Real-time PCR was conducted with Light Cycler (Roche Diagnostics GmbH) using SYBR green (TaKaRa Bio, Otsu, Shiga, Japan). The primers used were 5'-TGGAGTCACAGAAGGAGTGGCTAAG-3' and 5'-TCTGACCACAGTGAGGAATGTCCAC-3' for IL-6, 5'-GATGACCCAGATCATGTTTGA-3' and 5'-GGAGAGCATAG

CCCTCGTAG-3' for  $\beta$ -actin. All estimated mRNA values were normalized to  $\beta$ -actin mRNA levels.

**Enzyme-linked immunosorbent assay** RPE-choroid complexes were isolated from B6 mice at 2d after induction of subretinal fibrosis and immersed in 200  $\mu\text{L}$  tissue protein extraction reagent (T-PER; Pierce, Rockford, IL, USA), supplemented with protease inhibitor cocktail (Halt; Pierce). The mixture was homogenized (Polytron; Kinematica AG) and clarified by centrifuging at 10 000 g for 5min. IL-6 level in the lysate was determined by a mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

IL-6R monoclonal antibody treatment B6 mice received IL-6R monoclonal antibody (MR16-1, 10 mg/kg body weight in PBS, provided by Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) by intraperitoneal injection 1h before subretinal induction, and the treatments were continued daily until the end of the study. Control mice were treated with rat IgG (R&D Systems) at identical doses as those for MR16-1. Seven days later, the area of GFAP was measured to quantify the subretinal fibrosis on choroidal flat mounts, to assess the effect of the MR16-1 on experimental subretinal fibrosis.

**Statistical Analysis** Each result is representative of at least three independent experiments. All values are expressed as the mean  $\pm$  SD. Statistical analyses were made using Student's *t*-test (SPSS, Chicago, IL, USA) where appropriate.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Interleukin-6 Expression During Experimental Subretinal Fibrosis Development

The expression of IL-6 during the development of experimental subretinal fibrosis was measured by quantitative real-time RT-PCR assay. IL-6 mRNA level was significantly elevated ( $P < 0.01$ ) at 1d after subretinal fibrosis induction and increased further to about 12-fold at 2d ( $P < 0.01$ ), reaching the peak, and then substantially returned to baseline at 5d (Figure 1).

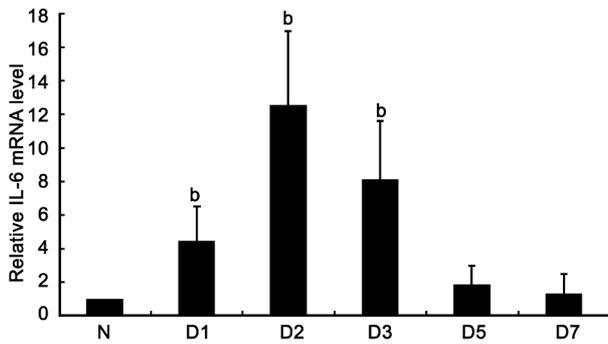
We next used ELISA analyses to detect IL-6 protein in RPE-choroid complexes after subretinal fibrosis induction. IL-6 protein was not detected in naive mice. At 2d after subretinal fibrosis induction, IL-6 protein level was upregulated to  $67.33 \pm 14.96$  pg/mg in subretinal fibrosis mice (Figure 2).

### IL-6R Monoclonal Antibody Suppresses Subretinal Fibrosis

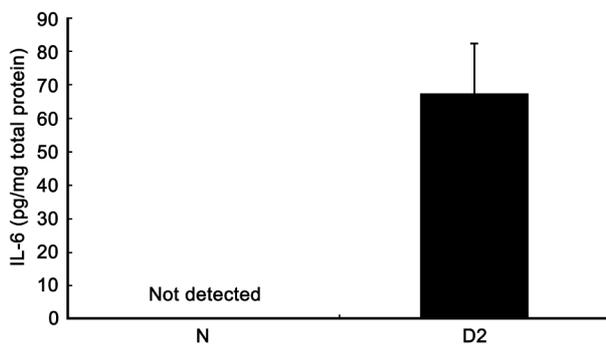
To address the roles of IL-6 signaling in this subretinal fibrosis model, we further investigated the effect of IL-6R monoclonal antibody (MR16-1) on subretinal fibrosis using intraperitoneal administration. MR16-1 resulted in a reduced subretinal fibrosis, and the GFAP-positive areas significantly decreased by 48% compared to animals from control group (Figure 3). No signs of systemic toxicity for intraperitoneal MR16-1 at this dosage (data not shown).

## DISCUSSION

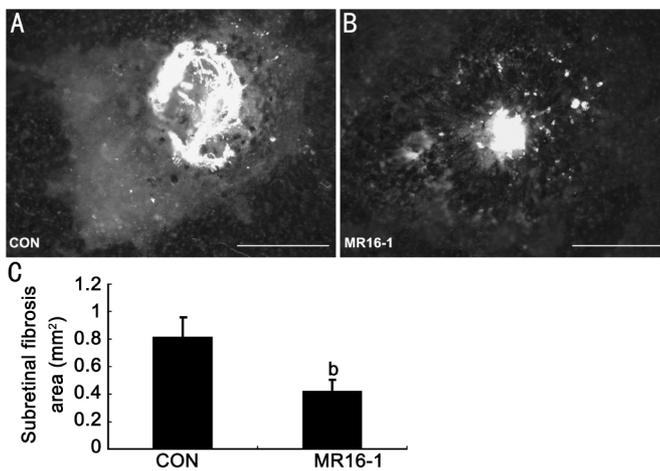
The present study reveals, for the first time to our knowledge, two important findings concerning the



**Figure 1 IL-6 mRNA expression in the course of subretinal fibrosis development** IL-6 mRNA expression in RPE-choroid complexes was determined by quantitative RT-PCR analysis at various time points after subretinal fibrosis introduction.  $n=6$  for each time point. <sup>b</sup> $P<0.01$ . Error bars: SD.



**Figure 2 IL-6 expression in the course of subretinal fibrosis development** The expression of IL-6 in RPE-choroid complexes was determined by ELISA at 2d after subretinal fibrosis introduction.  $n=8$  for each time point. Error bars: SD.



**Figure 3 Suppression of subretinal fibrosis by IL-6R blockade in murine model** Subretinal fibrosis was induced by subretinal injection of PECs following photocoagulation and immunofluorescence staining was performed on flatmounts from control group (A) or IL-6R monoclonal antibody (MR16-1)-treated (B) mice 7d later, with GFAP conjugated to FITC to visualize subretinal scar. Quantitative analysis was performed by measuring fluorescence-positive area (C). CON,  $n=28$ , MR16-1,  $n=28$ . Scale bar: 500  $\mu\text{m}$ . <sup>b</sup> $P<0.001$ . Error bars: SD.

relationship of the IL-6 signaling with subretinal fibrosis. First, IL-6 was present in experimental subretinal fibrosis and upregulated during subretinal fibrosis development.

Second, subretinal fibrosis was suppressed by blocking IL-6 signaling using IL-6R monoclonal antibody.

IL-6 is a 26-kDa glycopeptide whose gene is found on chromosome 7, signaling primarily through a protein complex including the membrane-bound, non-signalling  $\alpha$ -receptor subunit (IL-6R) and two signal-transducing gp130 subunits [15]. While gp130 is expressed ubiquitously, IL-6R is predominantly expressed on hepatocytes, neutrophils, monocytes/macrophages and some lymphocytes [16]. IL-6 is traditionally considered a regulator of acute phase inflammatory responses and a lymphocyte stimulatory factor. However, this cytokine is also involved in wound healing, angiogenesis and fibrogenesis. For example, it has been shown that IL-6 is a profibrogenic cytokine in systemic sclerosis (SSc)[10]. One case series has indicated that the use of tocilizumab, which blocks IL-6 trans-signalling, in 2 patients with diffuse cutaneous SSc, one with renal involvement and the other with lung fibrosis, resulted in a decrease in skin thickening. Skin biopsies taken before and after tocilizumab treatment indicated a reduction in collagen[17].

In ocular fibrotic responses, IL-6 has been previously detected in the subretinal fluid and the vitreous of patients with proliferative vitreoretinopathy (PVR)[18]. Vitreous cells have been shown to enhance the proliferation of vascular endothelial cells *via* production of IL-6 *in vitro*, thus contributing to the pathogenesis of proliferative vitreoretinal diseases [19]. IL-6R mRNA expression is also detected on cultured RPE cells [20], which having a central role in ocular fibrotic responses. In neovascular AMD, IL-6 might have dual pathogenic functions by promoting both angiogenesis and RPE degeneration in advanced AMD [21]. Increased IL-6 levels are found in ocular fluids of patients with neovascular AMD and they predict AMD progression [22]. The pro-angiogenic effects of IL-6 are well-described in the context of tumour angiogenesis and involve the upregulation of VEGF-A [23]. Genetic ablation of IL-6 or its receptor decreases laser-induced choroidal neovascularization [13]. On the other hand, IL-6 signalling also promotes degeneration of RPE following lipopolysaccharide stimulation [24]. In the present study, the expression of IL-6 was upregulated during the development of experimental subretinal fibrosis, and systemic blockade of IL-6R led to significant suppression of subretinal fibrosis. These results indicated that IL-6 signaling may contribute to the pathogenesis of subretinal fibrogenesis and late-stage neovascular AMD. As ideally treatment modalities for neovascular AMD would target the multiple mechanisms of AMD associated vision loss, including inflammation, neovascularization and fibrosis, our results suggest IL-6 as an attractive molecular target in the treatment of neovascular AMD.

In a previous study, we have demonstrated the important role of transforming growth factor (TGF)- $\beta$  in the pathogenesis of subretinal fibrogenesis, in which, TGF- $\beta$  neutralizing antibodies (NAb) treatment resulted in a reduced subretinal

fibrosis by 65%. The interaction between TGF- $\beta$  and IL-6 has been investigated in several studies. TGF- $\beta$  induces IL-6 production in some cell types including human fibroblasts, osteoblasts and prostate cancer cells [25]. The upregulation of IL-6 by TGF- $\beta$  has also been described in human RPE cells, which may provide one explanation for the similar results of TGF- $\beta$  NAb and MR16-1 treatment in the subretinal fibrosis inhibition [26].

In summary, we report for the first time that IL-6 was upregulated during subretinal fibrosis development and IL-6R inhibition decreases subretinal fibrosis size. The future of neovascular AMD management may require combined therapies, with several drugs acting on different mediators of CNV and fibrosis, such as VEGF, complement system, TGF- $\beta$  and IL-6. Thus, in light of the current results, IL-6R inhibition could be considered in this multivariate neovascular AMD treatment.

#### ACKNOWLEDGMENTS

**Foundation:** Supported by Liaoning Science and Technology Project (No.2013225303)

**Conflicts of Interest:** Cui W, None; Zhang H, None; Liu ZL, None.

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