Interleukin-6 plays a crucial role in the development of subretinal fibrosis in a mouse model

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ABSTRACT

Subretinal fibrosis has been recognized as a feature of an advanced stage of exudative age-related macular degeneration (AMD) that leads to irreversible loss of vision. This study was aimed at elucidating roles of interleukin-6 (IL-6) in the development of subretinal fibrosis. Immunohistochemistry (IHC) was performed with anti-human IL-6 antibody in surgically excised choroidal neovascular tissues from patients with exudative AMD. The area of subretinal fibrosis was measured in a mouse subretinal fibrosis model with injection of control small interfering RNA (siRNA) or IL-6 siRNA, or isotype control antibody or anti-IL-6 receptor antibody after peritoneal exudative cells (PECs) injection into the vitreous cavity. PECs derived from IL-6\textsuperscript{+} or IL-6\textsuperscript{-} mice were placed into the subretinal space of IL-6\textsuperscript{+} or IL-6\textsuperscript{-} mice. IL-6 was expressed in the stroma and retinal pigment epithelial (RPE) layer in the choroidal neovascular tissues. IL-6 knockdowndown or blocking of the IL-6 receptor suppressed the formation of subretinal fibrovascular scars. The area of subretinal fibrosis induced by PECs derived from IL-6\textsuperscript{-} mice was less than that induced by PECs from IL-6\textsuperscript{+} mice. The results suggested that IL-6, expressed by activated macrophages, is a crucial mediator that promotes subretinal fibrosis. Targeting IL-6 and the corresponding signalling pathway would be an attractive therapeutic approach not only in choroidal neovascularization, but also in subretinal fibrosis.

1. Introduction

Age-related macular degeneration (AMD) is the most common cause of severe vision loss in developed countries [1]. Most blindness in AMD results from choroidal neovascularization (CNV), which is the result of aberrant new vessels growing from the pre-existing choroidal vasculature, breaking through the Bruch’s membrane and extending into the subretinal pigment epithelial space and/or subretinal space [2]. The mechanisms of CNV are still poorly understood in most conditions; however, the extent to which a wound healing response contributes the initiation of a CNV response is completely unclear [3,4].

Tissue fibrosis is a common pathophysiological response to chronic injury and can be considered as a wound repair of damaged organs, mostly associated with a robust inflammatory response [5]. CNV causes hemorrhage and extravasation of plasma proteins, including those of the extracellular matrix, such as fibrinogen that clots to form a fibrin gel. Infiltration of inflammatory cells into the fibrin gel, the transdifferentiation of retinal pigment epithelial (RPE) cells into fibroblast-like cells via epithelial-mesenchymal transition (EMT) and an excessive and poorly ordered extracellular matrix deposition often result in the formation of subretinal fibrovascular tissue, which replaces the normal retinal structure including photoreceptors, within and/or around the macula area. Furthermore, subretinal fibrosis develops in approximately 50% of all treated eyes within two years post continuous anti-vascular endothelial growth factor (VEGF) treatment, which is the first choice for treatment in patients with wet AMD, which results in visual impairment [6,7]. Because patients with this condition show severe permanent visual loss, both the elucidation of the molecular mechanisms underlying tissue scar formation in CNV and the development of therapeutic strategies for tissue fibrosis are desired.
Several studies have reported that activated macrophages play a pivotal role in the development of tissue fibrosis in other organs such as the liver and kidney [8,9]. In the eye, macrophage infiltration is also thought to be an important pathological process in subretinal proliferation in retinal diseases, because macrophages can produce fibrogenic, proliferation and angiogenic factors [10]. Recently, we showed that injection of isolated peritoneal exudates cells (PECs) into the subretinal space can promote EMT in RPE cells and subsequently result in subretinal scar formation [11], which indicated that macrophages may trigger subretinal fibrosis.

Interleukin (IL)-6 is a pleiotropic cytokine that plays a role in biological processes such as immune response, inflammation, wound healing response and angiogenesis [12]. IL-6 binds to the IL-6–binding chain receptor (IL-6R) and the complex of IL-6 and IL-6R associates with signal-transducing chain gp130 to result in homodimerization of gp130 to trigger the IL-6 signaling pathway [13]. Then, the dimerization of gp130 leads to activation of the Janus kinase (JAK)–signal transducer and activator of transcription 3 (STAT3) and the JAK-extracellular signal transducer and activator dimerization of gp130 leading to the activation of the Janus kinase (JAK)–signal transducer and activator of transcription 3 (STAT3) and the JAK-extracellular signal-regulated kinase (ERK) pathway. Persistent aberrant IL-6 production has been implicated in the development of various autoimmune and chronic inflammatory diseases as well as in cancers [14]. IL-6 has also been found to be associated with many fibrotic diseases including idiopathic lung fibrosis, hepatocellular fibrosis, renal fibrosis, graft-versus-host disease (GVHD), liver fibrosis, keloids and systemic sclerosis [15]. In a previous study, analyses of mice deficient in IL-6 revealed an impaired wound healing response in several organs, including the liver and the skin [16,17]. However, this impaired cutaneous wound healing response in the IL-6 deficient mice was recovered upon IL-6 administration, indicating that IL-6 might be essential for wound healing [17]. In human AMD, high levels of IL-6 in the blood samples of patients with exudative AMD have been correlated with disease progression [18]. Moreover, in a murine laser-induced CNV model, IL-6 has been reported to induce VEGF and ccl2 expression in vascular endothelial cells, leading to the development of CNV [19]. Furthermore, transforming growth factor β2 (TGFβ2), which promotes the EMT of RPE cells [20], was found to induce subretinal fibrosis as well as IL-6 production in RPE cells [21]. Based on these findings, we predicted that the analysis of the relationship between subretinal fibrosis and IL-6 using our recently developed animal model could provide new insights into the mechanisms of subretinal fibrosis [11]. Therefore, in this study, we investigated the role of IL-6 in mice by genetic ablation and its potential as a therapeutic target using short-interfering RNA (siRNA) against IL-6 and neutralizing anti-IL-6 receptor antibody in a murine subretinal fibrosis model.

2. Materials and methods

2.1. Human tissue

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the Kyushu University Institutional Review Board for Clinical Research. We obtained written informed consent from all the participants before any study procedures or examinations were performed. CNV tissue was excised from patients with AMD who had not received previous AMD treatment at the Department of Ophthalmology, Nihon University Hospital. The diagnoses were confirmed by dilated ophthalmic examination prior to acquisition of the tissues or eyes. The study follows the guidelines of the Declaration of the Helsinki.

2.2. Mice

Female C57BL/6 J (IL-6−/−) mice and IL-6+/− mice with a C57BL/6 J background between 7 and 10 weeks of age were purchased from Japan SLC (Shizuoka, Japan) or Jackson Laboratories (Bar Harbor, ME, USA). All the animal experiments were approved by the committee on the Ethics of Animal Experiments, Kyushu University, Graduate School of Medical Sciences. All the animals were maintained in specific pathogen-free conditions at Kyushu University and treated humanely according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

2.3. Immunohistochemistry (IHC)

Paraffin-embedded sections of human eyes or surgically excised CNV tissues were deparaffinized and rehydrated with a graded alcohol series. Immunohistochemical staining with the primary antibodies specific for human IL-6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed using horseradish peroxidase. 3,3'-Diaminobenzidine (DAB) was used as a chromogen with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Hematoxylin (Dako, Glostrup, Denmark) was used for counterstaining.

2.4. Preparation of PECs

Macrophage-rich peritoneal exudative cells (PECs) were obtained from mice that received 2.5 mL of 3% aged thioglycolate solution (Difco, Detroit, MI, USA) three days before killing. The PECs were
placed in 10 cm dishes, from which the nonadherent cells were washed out using phosphate buffered saline (PBS). Next, the remaining adherent cells were incubated with 0.02% ethylenediaminetetraacetic acid (EDTA) and were collected by vigorous pipetting. The cells were then counted and resuspended in PBS.

2.5. Mouse model of subretinal fibrosis

Mouse models of subretinal fibrosis were established as described previously [11]. Briefly, laser photocoagulation (wavelength 630 nm, 0.1 s, spot size 75 μ, and power 200 mW) was performed in the posterior pole of retina around the disc to create a subretinal bubble and rupture Bruch’s membrane. PECs (4×10⁶) collected as described above without any stimulation were then inoculated into the subretinal space. Neutralizing anti-mouse IL-6 receptor antibody (MR16-1) which was kindly provided by Chugai Pharmaceutical Co. (Tokyo, Japan) or an isotype-matched control rat IgG was injected into the vitreous cavity two hours after PEC-inoculation. On day 7 after PEC-inoculation, the mice were killed and their eyes were enucleated; the eyes were then fixed in 4% paraformaldehyde. Next, choroid flatmount were prepared and stained with anti-glial fibrillary acidic protein (GFAP) antibody conjugated with fluorescein isothiocyanate (FITC) (Dako). GFAP was used as a marker of subretinal fibrosis in this model, because the area that glia cells accumulated in coincided with the subretinal fibrosis area stained with collagen type I, a direct marker of fibrosis. However, direct collagen type I staining could not constantly detect the fibrotic area in this model [11]. Images were captured using a fluorescence microscope (DMIRE2; Leica). GFAP-positive area was measured using Image J software (NIH, Bethesda, MD, USA).

2.6. Intravitreal injection of siRNA

IL-6-targeting siRNA and control siRNA were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The siRNAs were resolved in siRNA suspension buffer and were injected into the vitreous cavity just after PEC inoculation in the subretinal fibrosis model.

2.7. Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) from the eyes of wild type (WT) mice treated with either IL-6-targeting siRNA or control siRNA at day 14 after immunization. Aliquots containing 1 μg of total RNA were then reverse transcribed using a RT-PCR kit (First-Strand complementary DNA (cDNA) Synthesis Kit; Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer’s instructions. Next, the cDNA samples were subjected to 40 cycles of amplification with pre-designed TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for each target (IL-6: Mm00446190_m1) in a LightCycler Instrument (Roche Diagnostics) and was quantified by the comparative cycling threshold method. Estimated mRNA values were then normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) levels.

2.8. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) and Scheffe’s test. p < .05 was considered statistically significant. Each histogram represents the mean ± standard deviation (SD) unless otherwise noted.

3. Results

3.1. IL-6 expression was detected in the human fibrotic tissue obtained from AMD patients

IL-6 levels in the aqueous humor in patients with exudative AMD are significantly increased compared to those in the controls [22]. To assess the expression of IL-6 histologically, we performed IHC for negative control or anti-IL-6 antibody on surgically excised choroidal fibrovascular membranes from a patient with exudative AMD who had not received previous AMD treatment. In IHC with the negative control, a partially intact monolayer of RPE cells was observed in the patient (Figure 1(A)). IL-6 was expressed in the stroma cells and an intact monolayer of RPE cells in the examined specimen (Figure 1(B)).

3.2. Neutralization of the IL-6 signaling pathway reduced subretinal fibrosis

Initiation of fibrosis is induced by activated fibroblasts that produce excess collagen and extracellular matrix [23]. The collagen synthesis is induced by the stimulation of IL-6 in hepatic inflammation [24]. IL-6 also enhances the production of tissue inhibitor of metalloproteinases (TIMP), which inhibit enzymatic activity to digest the extracellular matrix [25]. To assess whether the IL-6 signaling pathway involving the IL-6 receptor promoted subretinal fibrosis, we treated the eyes with the anti-IL-6R antibody (MR16-1) in order to inhibit IL-6 signaling in the eyes of mice with subretinal fibrosis. The area of
subretinal fibrosis in anti-IL-6 R antibody-treated mice was significantly lower than that in the control antibody-treated mice (Figure 2).

3.3. IL-6 knockdown reduced subretinal fibrosis

Next, to assess the effect of endogenous IL-6 on subretinal fibrosis, we suppressed IL-6 synthesis by using IL-6 siRNA. First, we defined the effective concentration of IL-6 siRNA required to suppress the expression of the IL-6 gene in the eyes of mouse models of subretinal fibrosis. Compared with control siRNA, 49 and 73 ng of IL-6 siRNA significantly reduced the expression of the IL-6 gene in the eyes (Figure 3(A)). We then performed treatment with 73 ng of IL-6 siRNA or control siRNA in the subretinal fibrosis model and found that the area of subretinal fibrosis in mice injected with IL-6 siRNA was significantly reduced when compared with the mice injected with control siRNA (Figure 3(B)).

3.4. Subretinal fibrosis was suppressed upon administration of IL-6/C0 PECs

To investigate whether IL-6 derived from macrophages promoted subretinal fibrosis, we injected IL-6/C0+ or IL-6/C0- PECs into the subretinal space of IL-6/C0+ mice. The area of subretinal fibrosis induced upon injection of IL-6/C0- PECs was significantly decreased when compared with that induced upon injection with of IL-6/C0+ PECs (Figure 4(A)). These results suggested that IL-6 derived from macrophages played a pivotal role in subretinal fibrosis.

4. Discussion

In this study, we found the IL-6 expression in the stroma and monolayer of RPE cells in patients with exudative AMD. Most of the stromal cells were immunoreactive for both alpha-SMA and cytokerin, as determined using IHC [26]. In addition, because of a gradient change of RPE cells from cytokekin-positive, mildly alpha-SMA-positive cells adjacent to the normal RPE layer to non-pigmented cytokekin-negative, alpha-SMA-positive stroma cells and to cytokekin-negative, alpha-SMA-positive cells in the stroma, we hypothesized that myofibroblast in human choroidal fibrovascular tissues originate from RPE cells [26]. Cui et al. [27] reported that IL-6 is increased in the RPE-choroid lesion and that inhibition of IL-6 signaling can suppress subretinal fibrosis in the murine subretinal fibrosis model. These results suggest that IL-6 derived from RPE cells is essential for the development of human choroidal fibrovascular tissues.

In addition, infiltration of CD68-positive macrophages is reported to be abundant in the stroma as well as in RPE cells [28]. Our previous study as well as other previous reports have shown that ocular-infiltrating macrophages play a pivotal role in the development of CNV via CCR2, the receptor of ccl2, in a laser-induced CNV model [29,30]. In the laser
induced CNV model, IL-6 was reported to be able to recruit macrophages into laser-burned sites through the induction of ccl2 [19]. In an analysis of ccl2/C0 mice in our subretinal fibrosis model, we revealed that ccl2 is essential to promote the accumulation of macrophages in the laser-burned area, leading to the development of subsequent subretinal fibrosis [11]. Therefore, lack of IL-6 from injected macrophages reduced the subretinal scar formation in vivo, which suggested that IL-6 produced by infiltrating macrophages play a primary role in ccl2 induction in subretinal fibrosis as well as in CNV.

EMT, which is characterized by the loss of cell-cell junctions and cell polarity, is one of the pivotal processes during the development of subretinal scar formation [6]. We previously showed that transforming growth factor-beta 2 (TGF-β2) not only promotes the EMT through the upregulation of alpha-SMA, vimentin and fibronectin, but also promotes IL-6 production in RPE cells [21]. It has also been reported that IL-6 synergistically acts with TGF-β2 to promote EMT through the JAK2/STAT3 pathway and subsequent activation of mothers against decapentaplegic homolog 3 (Smad3) in lung cancer cells [31]. IL-6 has also been found to directly induce EMT through the JAK/STAT3/Snail pathway in lung cancer cells [32]. Although the expression of IL-6 R is limited in specific cell lineages, RPE cells express the IL-6 R gene [33], which suggests that IL-6 mediates the EMT in RPE cells, not only in an autocrine loop, but also in a paracrine loop with TGF-β2.

Several researchers also have reported that activation of STAT3 is associated with renal fibrosis and lung fibrosis [34,35]. However, during a wound healing response in the skin, IL-6 induces alpha-SMA expression via the activation of JAK1 and ERK, but not STAT3 phosphorylation, in dermal fibroblasts [36]. Recently, it has been reported that IL-6 R is limited in specific cell lineages, RPE cells express the IL-6 R gene [33], which suggests that IL-6 mediates the EMT in RPE cells, not only in an autocrine loop, but also in a paracrine loop with TGF-β2.

Figure 3. Effects of knockdown of the IL-6 gene on subretinal fibrosis. (A) Expression of the IL-6 gene was assessed in the eyes of WT mice injected with IL-6 siRNA in a dose-dependent manner and were compared with the results for the scramble siRNA-injected eyes in the subretinal fibrosis model (n = 3). (B) The area of subretinal fibrosis was decreased upon intravitreal injection of IL-6-siRNA compared with that observed upon injection of control-siRNA (n = 3). The data are shown as means ± SD. The experiment was repeated three times with similar results. *p < .05.

Figure 4. Subretinal fibrosis upon administration of IL-6−/− PECs. The area of subretinal fibrosis in the eyes of the WT mice was reduced upon the subretinal injection of IL-6−/− PECs when compared with that observed upon injection of IL-6+/+ PECs (n = 3). The data are shown as means ± SD. The experiment was repeated three times with similar results. *p < .05.
In summary, our results from the present study indicate that IL-6 plays a pivotal role in the development of subretinal fibrosis. Numerous studies on the mechanisms of CNV have been performed and the accumulated knowledge has led to the development of therapies for exudative AMD, such as anti-VEGF therapy, verteporfin photodynamic therapy and combined therapy [38]. Recently, therapeutic strategies for the prevention of subretinal fibrosis in patients with exudative AMD has also been initiated synergistically with anti-CNV therapy by targeting platelet-derived growth factor BB [6]. Therefore, we propose that anti-IL-6 therapy could be effective for both CNV and subretinal fibrosis and that it will help efficiently achieve better visual prognosis in patients with exudative AMD, although it is necessary to confirm its long-term safety and impaired wound healing response in the retina. The potential of therapeutic targeting against subretinal fibrosis also warrants further research in this area.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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