Interleukin-13 and age-related macular degeneration

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Abstract

AIM: To identify the effects of interleukin (IL)-13 on retinal pigment epithelial (RPE) cells and the IL-13 level in aqueous humor of age-related macular degeneration (AMD) patients.

METHODS: IL-13 levels in aqueous humor specimens from AMD patients were detected with enzyme-linked immunosorbent assay (ELISA). ARPE-19 cells were treated with 10 ng/mL IL-13 for 12, 24, and 48h. The cell proliferation was evaluated by the MTS method. The mRNA and protein levels of α-SMA and ZO-1 were evaluated with quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot respectively. The expression of tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) were assessed by ELISA.

RESULTS: IL-13 levels in the aqueous humor of patients with AMD were significantly higher than those in the control (167.33±17.64 vs 27.12±5.65 pg/mL; P=0.01). In vitro, IL-13 of high concentrations (10, 15, and 20 ng/mL) inhibited ARPE-19 cell proliferation. α-SMA mRNA in ARPE-19 cell increased (1.017±0.112 vs 1.476±0.168; P<0.001) and ZO-1 decreased (1.051±0.136 vs 0.702±0.069; P<0.001) after treated with 10 ng/mL IL-13 for 48h. The protein expression of α-SMA and ZO-1 also showed the same tendency (α-SMA: P=0.038; ZO-1: P=0.008). IL-13 significantly reduced the level of TNF-α (44.70±1.67 vs 31.79±3.53 pg/mL; P=0.005) at 48h, but the level of TGF-β2 was significantly increased from 34.44±2.92 to 57.61±6.31 pg/mL at 24h (P=0.004) and from 61.26±1.11 to 86.91±3.59 pg/mL at 48h (P=0.001). While expressions of VEGF didn’t change after IL-13 treatment.

CONCLUSION: IL-13 in vitro inhibit ARPE-19 cell proliferation and expression in the aqueous may be associated with AMD.

KEYWORDS: interleukin-13; age-related macular degeneration; retinal pigment epithelial cell

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INTRODUCTION

Age-related macular degeneration (AMD) is one of the most common causes of blindness in elderly individuals in the developed world[1]. AMD is typically categorized into two forms: wet form and dry form. The pathology of neovascular (wet) AMD is related to the process of choroidal neovascularization (CNV), which leads to leak exudates and hemorrhage. The neovascular membrane in the macular region becomes fibrotic and forms a disciform scar in the involutional stage, which causes irreversible damage and explains the severe central visual loss experienced by these patients[2]. While molecular and cellular mechanisms underlying CNV are complicated and not fully elucidated, local inflammation and angiogenesis, which influence disease development and prognosis, are recognized as key factors in the progression of wet AMD.

Current clinical strategies for treating progressive AMD are primarily aimed at inhibiting vascular endothelial growth factor (VEGF), an endothelial cell mitogen shown to be a key mediator of angiogenesis and a vascular permeability-enhancing factor. Therefore, only 30% to 40% treated patients gain three lines in visual acuity, some patients continue losing visual acuity, even progressing to blindness[3]. The greater fibrotic response after ranibizumab injection during wound healing process may contribute to poor prognosis[4,5]. Thus, to explore cytokines related to fibrosis may provide potential novel treatments of AMD.

Interleukin (IL)-13 plays pathophysiological roles in allergic inflammation and fibrosis formation. In this study, we quantified IL-13 levels in the aqueous humor of patients with AMD to identify its roles in the pathological process of this condition and investigated its relationship with VEGF, tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β)[6]. Identification of these cytokines has enabled the exploration of novel therapeutic approaches.

SUBJECTS AND METHODS

The study protocol was approved by the Ethics Committee of the institute and adhered to the guidelines of the Declaration of Helsinki. Informed consent was obtained from all patients and their families before enrollment.
**Human Aqueous Humor Specimens** Aqueous humor specimens from 52 patients with a confirmed diagnosis of AMD treated with intravitreal ranibizumab injections from October 2013 to July 2014 were analyzed. Twelve eyes with clinically apparent fibrotic disciform lesion on examination were included. The study’s inclusion criteria were as follows: 1) subretinal or vitreous hemorrhage; 2) absence of other evident causes of vasculopathy and exudative retinopathy, such as serious heart, lung, liver, or kidney dysfunction; 3) absence of other ocular diseases such as autoimmune uveitis, proliferative diabetic retinopathy, retinal vein branch obstruction, and pathological myopia. As controls, aqueous humor specimens, taken from the eyes of 20 patients undergoing cataract surgery, were also analyzed. There was no history of ocular operation or intravitreal ranibizumab treatment in control cases.

**Sample Collection** Aqueous humor specimens (0.05-0.1 mL) were collected before each intravitreal ranibizumab injection of patients with AMD. Similarly, aqueous samples (0.05-0.15 mL) were taken in the beginning of routine cataract surgery as a control group. These aqueous samples were transferred directly into Eppendorf tubes on ice, centrifuged at a speed of 2000 rpm for 10min, aliquoted, and stored at -80°C until analyzed.

**Cell Culture** Human retinal pigment epithelial (RPE) cell line ARPE-19 (ATCC, USA) was cultured using 1640 medium (Hyclone, USA) supplemented with 10% (v/v) fetal bovine serum (Hyclone, USA) and 1× solution of penicillin/streptomycin (Genview, USA). Cells were grown at 37°C, in a humidified atmosphere of 5% CO₂ and 90% relative humidity.

**MTS Proliferation Assay** The number of viable cells was measured using the MTS assay, which relies on the formation of a colored substrate by mitochondrial enzyme activity in viable cells. ARPE-19 cells were seeded at a density of 2×10⁶ cells per well on a 96-well plate and incubated at 37°C overnight and another overnight in serum-free media at 37°C. The cells were incubated in 2% FBS medium with or without IL-13 (1, 5, 10, 15, 20 ng/mL; Pepro Tech), at 37°C for 12, 24 or 48h. MTS (20 μL per well) was then added for 3h. The absorbance at 490 nm was determined using a plate reader (iMark, Bio-rad, USA). Each experiment was conducted at least four replicates.

**Quantitative Real-time Polymerase Chain Reaction** ARPE-19 cells were seeded in 6-well tissue culture plates in amounts of 1×10⁵ cells for incubation of 24h and serum-starved overnight and then cultured in 2% FBS with 10 ng/mL IL-13 treatment for 12, 24, and 48h. The total RNA was extracted using RNA extraction kit (Takara, Japan) according to the manufacturer’s instructions and was reverse-transcribed using PrimeScript RT reagent Kit (Takara, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with SYBR Green RealMasterMix and specific primers for α-SMA (forward, 5'-GGGACATCAAGGAGAACACTGTGT-3'; reverse, 5'-TCTCTGGGCAGCGGAAC-3') and ZO-1 (forward, 5'-TTCCAGATAAAAGCCCGAGTTAAG-3'; reverse, 5'-GCAGAAGATTGTGATTGAGT-3'). GAPDH was used as an endogenous standard to estimate the mRNA levels. The PCR programme was designed as follows: the cDNA was denatured at 95°C for 30s followed by 40 cycles of 95°C for 15s, 60°C for 30s and 72°C for 30s.

**Western Blot** ARPE-19 cells were seeded in 25-cm² tissue culture flask in amounts of 4×10⁵ cells for incubation of 24h and serum-starved overnight and then cultured in 2% FBS with 10 ng/mL IL-13 treatment for 48h. Cells were harvested in 120 μL 1.5× loading buffer. Samples were clarified by centrifugation at 12 000 rpm at 4°C for 30min. The homogenates, which contained 30 μg of protein, were then separated by SDS-PAGE and transferred into nitrocellulose membrane (Beyotime, Beijing, China). The blots were probed with the following primary antibodies: α-SMA (Abcam Cambridge, UK; 1:500), ZO-1 (Abcam Cambridge, UK; 1:1000), GAPDH (Abcam Cambridge, UK; 1:5000). Quantification of the Western blot data was performed by measuring the intensity of the hybridization signals using Image J software, and the ratios of α-SMA and ZO-1 to GAPDH were used as the relative expression level of the target protein.

**Enzyme Linked Immunosorbent Assay** After experimental incubations as mentioned above, cell culture supernatants were collected and centrifuged for 10min at 1000 g (4°C) to remove cell debris, then stored at -80°C until use. The amount of secreted cytokines (TNF-α, TGF-β2 and VEGF) were examined using commercial ELISA kits (eBioscience, USA) according to the manufacturer’s protocol. The plates were read at 450 nm, and cytokine levels were extrapolated from standard curves and normalized to protein concentrations.

**Statistical Analysis** All data from quantitative assays were expressed as the mean±standard deviation (SD). At least 2-3 independent repetitions with triplicate determinates were performed for each quantitative assay. Statistical analysis was performed using one-way ANOVA (Tukey’s post hoc test was used to analyze differences between parametric groups) or Student’s t-test (independent sample t-test) with the aid of SPSS17.0. A significance cutoff of P<0.05 was used.

**RESULTS**

**Clinical Characteristics of Subjects** Aqueous humor specimens from 52 patients (60 eyes; 27 men and 25 women; mean age, 62.05±7.06y) with a confirmed diagnosis of AMD treated with intravitreal ranibizumab injections from October 2013 to July 2014 were analyzed. Mean visual acuity was 0.25 preoperatively (range 0.02-0.6) and 0.37 postoperatively (4wk after injection, range 0.05-0.8). As controls, aqueous humor specimens, taken from the eyes of 20 patients (20 eyes; 10 men and 10 women; mean age, 61.63±7.58y) undergoing
cataract surgery, were also analyzed. There were no significant differences in the distribution of age and gender between the two groups (P>0.05).

**Interleukin-13 Level in Aqueous Humor** IL-13 levels in the aqueous humor of patients with AMD were significantly higher than those in the control (167.33±17.64 vs 27.12±5.65 pg/mL; P<0.01) (Figure 1).

**MTS Proliferation Assay** After the ARPE-19 cells were incubated with different doses of IL-13 for 48h, the optical density (OD) values, which reflect cell number, were not significantly different in the low concentration of IL-13 (1 and 5 ng/mL) treatments compared with the control group, while cellular viability decreased significantly of increasing concentration of IL-13 (10, 15, and 20 ng/mL) groups. As shown by Tukey's post hoc tests (Figure 2), relative OD values were significantly decreased in groups treated with 10 ng/mL (12h, 0.76±0.08; 24h, 0.70±0.11; 48h, 0.72±0.05; P<0.01), 15 ng/mL (12h, 0.81±0.05; 48h, 0.83±0.07; P<0.01) and 20 ng/mL (48h, 0.84±0.05; P<0.01).

**Effect of Interleukin-13 on α-SMA and ZO-1 Expression in ARPE-19 Cells** Epithelial-mesenchymal transition (EMT) is characterized by loss of epithelial makers such as ZO-1 and replacement by mesenchymal markers as α-SMA. After ARPE-19 cell were incubated with IL-13 treatment for 48h, the mRNA levels of α-SMA and ZO-1 were not affected at 12 and 24h. In contrast, α-SMA mRNA increased (1.017±0.112 vs 1.476±0.168, P<0.001) and ZO-1 decreased (1.051±0.136 vs 0.702±0.069, P<0.001) at 48h (Figure 3A). Meanwhile, the α-SMA and ZO-1 protein expression also showed the same tendency in Western blot assay (α-SMA: P=0.038; ZO-1: P=0.008) (Figure 3B).

**Effects of Cytokines Induced by Interleukin-13** ARPE-19 cells were pretreated either with IL-13 or vehicle only and following 12, 24 and 48h of activation, conditioned media were analyzed by ELISA for levels of TNF-α, TGF-β2 and VEGF-A. IL-13 significantly reduced the level of TNF-α (44.70±1.67 vs 31.79±3.53 pg/mL; P=0.005) at 48h (Figure 4A). However, the level of TGF-β2 was significantly increased from 34.44±2.92 to 57.61±6.31 pg/mL at 24h (P=0.004) and from 61.26±1.11 to 86.91±3.59 pg/mL at 48h (P<0.001) (Figure 4B). While there was no significant difference of VEGF-A level between IL-13 group and control group (Figure 4C).

**DISCUSSION**

The advent of treatments focusing on CNV suppression has caused a major shift in the approach to disease therapy and prevention. Along with the development of molecular biology technology and cell biology, investigation into the roles of cytokines in the pathogenesis of CNV has progressed. The advent of intravitreous VEGF inhibitors has revolutionized the management of neovascular AMD[7]. Although these available treatments may limit CNV progression to a certain extent, a
portion of CNV patients still experience clinical deterioration. Furthermore, the loss of VEGF neuroprotecivity can be a potential side effect\cite{8-10}. Therefore, new antiangiogenic therapies for CNV are eagerly awaited. Our results support the previous viewpoints.

IL-13, a cytokine mainly produced by type 2 helper T cells and monocytes/macrophages, plays pathophysiological roles in allergic inflammation and fibrosis formation, and more recently has been shown to play a pivotal role in a number of fibrotic diseases including hepatic and pulmonary fibrosis, and nodular sclerosing Hodgkin’s disease\cite{11-12}. In this study, we found that IL-13 of aqueous humor was significantly upregulated during AMD development, suggesting that IL-13 could have potent effects on the pathological process of this disease. During chronic inflammation, IL-13 is induced concomitantly with activation of its downstream signaling via an IL-13 receptor, IL-13 Rα2\cite{13}. Concentrations of IL-13 increased in vitro could inhibit ARPE-19 cell proliferation in our study. The degeneration and decrease in cell viability of RPE cell are mostly contributed to process of AMD\cite{14-15}. We also found that IL-13 (10 ng/mL) can promote EMT in ARPE-19 cells. Greater fibrotic responses after IL-13 may result from an imbalance in the complex interactions between angiogenesis and pro-fibrotic signaling pathway. In the development of AMD, the formation of neovascular membrane fibrosis leads to the development of disciform scars, which indicate irreversible damage\cite{16}. Meanwhile, there is no effective therapy for the associated symptoms once central vision decreases because of metamorphosis and scotoma caused by the scarring. IL-13 is an indispensable mediator of fibrosis\cite{17}. Recently, it was discovered that TGF-β, which contribute to the pathogenesis of subretinal fibrogenesis and angiogenesis, promotes the differentiation of fibroblasts into myofibroblasts, and can be regulated by IL-13\cite{18}. TGF-β is of primary importance in the development of AMD as it is a strong inducer of extracellular matrix synthesis and accumulation\cite{19}. Moreover, TGF-β can induce the transformation of RPE cells into fibroblast-like cells in vitro\cite{20}. TGF-β2, as a number of TGF-β family, is the main isoform of TGF-β in the eye and it is produced locally\cite{21-23}. In this study, we found IL-13 could significantly upregulate the concentrations of TGF-β2 in ARPE-19 cells. Similar findings have been shown that IL-13 induces lung fibrosis by selectively stimulating TGF-β\cite{24-25}. Blockage of TGF and IL-13 receptors prevents the progression of fibrosis\cite{26-28}. In this study, we also found IL-13 could inhibit TNF-α. TNF-α, as a potent proinflammatory cytokine implicated in tissue damages, mediates RPE cell dysfunction and plays an important role in the pathology of AMD\cite{29-30}. TNF-α increases the expression of VEGF and promotes macrophage-RPE migration\cite{31-32}. Accumulating evidence supports the theory that other factors, such as IL-13, also play a crucial role in the development of AMD\cite{33-34}. This study suggested that the possible mechanism of IL-13 in AMD: to promote the transformation from chronic inflammation to fibrogenesis by decreasing TNF-α and upregulating TGF-β. In addition, reports on the effects of IL-13 on angiogenesis have been controversial. Some studies suggest that IL-13 inhibits tube formation and endotheliocyte migration\cite{34}. IL-13 also reportedly suppresses the expression of VEGF and attenuates vascular tube formation via the JAK2-STAT6 pathway\cite{35}. Haas et al\cite{36} found that IL-13 an in vivo antiangiogenic factor and provide a rationale for its use in rheumatoid arthritis to control pathologic neovascularization. Nevertheless, our study in vitro showed that IL-13 couldn’t affect the accumulation of VEGF in ARPE-19 cells.

The present study reveals the relationship of IL-13 with AMD. Therapys targeting VEGF alone already prove inadequate. Focusing on IL-13 and other factors involved in disciform scarring, which prolongs the time for effective treatment, can bring about a major shift in the approach to disease treatment and prevention. This potential bio-target may also improve prognosis assistant with anti-VEGF treatments. However, this study did not investigate the relationship between the size or extent of disciform scarring and IL-13 levels. This needs to be clarified in further research.

In summary, our study showed that concentrations of IL-13 increases in aqueous humor of patients with AMD and in vitro inhibited ARPE-19 cell proliferation, suggesting that IL-13 may play important roles in the pathogenesis of neovascular
AMD. Furthermore, the possible mechanism of IL-13 in the pathogenesis of AMD was investigated that IL-13 may promote the transformation from chronic inflammation to fibrogenesis by decreasing RPE cell viability and regulating concentrations of related cytokines. These findings may provide evidence to support the development of future AMD therapies.

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