• Basic Research •

Age-related pro-inflammatory and pro-angiogenic changes in human aqueous humor

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Abstract

• AIM: To reveal age-related aqueous cytokine changes in human aqueous humor.

• METHODS: Aqueous humor was collected from 12 young children (3-6.5 years old) and 71 healthy adults (22-106 years old) with cataract but without other systemic or ocular disorders. Levels of 22 cytokines, chemokines and vascular endothelial growth factor (VEGF) were measured and analyzed.

• RESULTS: The following proteins showed significant increase from childhood to adult: interferon-gamma (IFN-γ), interleukin (IL)-13, IL-6, IL-12(p70), IL-10, CCL2, CCL3, CCL4, CXCL8, CXCL9, CXCL10, IFN-α2 and VEGF (all $P<0.05$). IFN-γ, IL-13, IL-12(p70), IL-10, CCL3, CXCL9 and VEGF also showed moderate strength age-related increase in the adult group ($r>0.5$). The strength of correlation between aging and CCL4 were fair ($r=0.398$). The concentrations of IL-2, IL-4, IL-5, IL-1β and TNF-α were low in both groups.

• CONCLUSION: From childhood to adult, the immunological milieu of the anterior chamber become more pro-inflammatory and pro-angiogenic. Such changes may represent the parainflammation state of the human eye.

• KEYWORDS: aqueous humor; cytokines; aging; parainflammation; macrophage; vascular endothelial growth factor

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INTRODUCTION

The aging of the immune system, also called immunosenescence, is featured by changes of both innate and adaptive immunity. Typical features of immunosenescence of the adaptive immunity are decreased number of naive T and B cells and the compensatory increase of memory T cells and antigen-experienced B cells[1]. Changes of the innate immunity are more diverse and organ-specific. Decreased Toll-like receptor expression in leukocytes, reduced chemotaxis, phagocytosis and intracellular killing and release of cytokines and granules of the granulocytes have been reported[2].

Immunosenescence often display tissue or organ specific features. Aging is associated with the development of parainflammation, a term which describes a beneficial and controlled low level of inflammation in response to accumulating tissue stress such as oxidative stress and chemical stress[3]. In retina, parainflammation is accompanied by changes of the neural retina, the retinal pigmented epithelium (RPE) and the choroid tissue[4-5]. The disruption of the delicate parainflammatory status, induced either by excess tissue stress buildup or genetic susceptibility, leads to frank inflammation which is responsible for the development of many age-related retinal diseases such as age-related macular degeneration and diabetic retinopathy[6].

Compared to the retina, little is known about the parainflammation of the anterior segment. Aqueous humor is an important immunological milieu of the anterior segment. In this study, we measured cytokine concentrations in aqueous samples obtained from adult and young child cataract patients, and analyzed age-related cytokine concentration changes in the adult cataract group.

SUBJECTS AND METHODS

Aqueous Sample Collection This study was performed in accordance to the tenets of the Declaration of Helsinki and approved by the Institutional Review Board (IRB) of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. Written consent was obtained from each participating patient or their guardians without stipend.

Consecutive patients elective for cataract surgery from 1st January 2008 to 1st January 2011 were enrolled in the study. Patients having any of the following conditions were excluded: systemic inflammatory or autoimmune diseases, currently taking immunosuppressive medications, diabetes, hypertension, had pre-existing ocular diseases (other than

Table 1 Demographic information of cataract patients participated in this study mean±SD (range)

Demographic information	Child	Adult
No. of patients	12	71
Male/Female	8/4	33/38
Age (mean±SD, y)[a]	4.67±0.33 (3.0-6.5)	68.45±2.04 (22-106)
Axial length (mean±SD, mm)[a]	22.78±0.39 (20.33-24.81)	25.33±0.41 (19.53-34.00)
Duration of cataract (y)	4.83±0.97	5.53±1.67

[a]$P<0.05$ between child and adult cataract subjects by ANOVA analysis.

cataract) or ocular surgery within 12-months. All patients denied past history of uveitis. The definition for hypertension was according to the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure (JNC 7)[7]. The young child patients enrolled all had congenital cataract but without other ocular complications such as glaucoma or retinal abnormalities.

About 50-100 μL of aqueous was aspirated into a sterile tuberculin syringe at the beginning of cataract surgery through the paracentesis entry. Samples were quickly spun at 300 g for 5min at 4℃ using a tabletop microcentrifuge and the supernatant was stored at -80℃ until further analysis.

Cytokine Analysis Bio-Plex Pro™ magnetic color-bead-based multiplex assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to measure the concentrations of the following human cytokines: interleukin-1 receptor antagonist (IL-1Ra), interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-17, interferon-alpha2 (IFN-α2), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), CCL2 (MCP-1, monocyte chemotactic and activating factor), CCL3 (MIP-1α, macrophage inflammatory protein-1α), CCL4 (MIP-1β, macrophage inflammatory protein-1β), CCL5 (RANTES, regulated on activation, normal T cell expressed and secreted), CCL11 (EOTAXIN), CXCL8 (IL-8), CXCL9 (MIG, monokine induced by interferon-gamma), CXCL10 (IP-10, interferon-gamma-induced protein 10 kDa) and vascular endothelial growth factor (VEGF). The assay was conducted according to the manufacturer's instruction. Fifty microliters (50 mL) of aqueous humour sample was used in each reaction. Fluorescence intensity (FI) from the immunoassay was acquired and analyzed using Bio-Plex™ 200 System (software version 6.0, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical Analysis Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) Version 19 (IBM Corporation, Armonk, NY, USA). Statistical significance was accepted at $P\leq0.05$. Age and axial length differences were compared by analysis of variance (ANOVA). Mann-Whitney *U* test was used to test the difference in cytokine concentrations between groups. Pearson binary correlation was performed to determine the association between age and each individual cytokine in the adult group.

RESULTS

Patient Information The demographic information of all patients participated in this study is listed in Table 1. Eighty-three patients, including 12 congenital cataract patients the age of 3 to 6.5 years old, and 71 adult cataract patients at the age of 22 to 106 years old were enrolled. All child patients had congenital cataract. All adult cataract subjects were of good general health and had no other ocular conditions for at least 6mo prior to the cataract surgery. The reported duration of cataract history refer to the time between the date the subject was first diagnosed as having cataract to the date the surgery was performed. The date of first diagnosis of cataract was mostly based on subject's recollection.

Aqueous Cytokine Contents To delineate a basic inflammatory profile of the aqueous, we measured the concentrations of a total of 22 cytokines, chemokines and VEGF in both adult and child aqueous samples (Table 2). These factors covered the basic components of Th1, Th2, macrophage, and other leukocyte secreted/responsive cytokines and chemokines. We found very low concentrations of IL-1β, TNF-α, IL-2, IL-4 and IL-5 in both adult and child aqueous samples. Most cytokines measured exhibited significantly higher concentrations in the adult aqueous than in the child's. These included IFN-γ, IL-6, IL-13, IL-12(p70), IL-10, IFN-α2, CCL2, CCL3, CCL4, CXCL8, CXCL9, CXCL10 and VEGF. IL-17 was the only cytokine which showed decreased concentration in the adult aqueous compared to child's.

Age-associated Aqueous Cytokine Changes in the Adult Group Differences in cytokine concentrations between young children and adults may reflect the maturation of the aqueous immune milieu. In order to know if aging is associated with changes in the aqueous cytokine concentration in the adults, we performed partial correlation analysis between age and individual cytokine concentrations controlling for both axial length and the duration of cataract. The cytokines which showed significant correlation were given in Table 3. Correlation of moderate strength ($r>0.5$) was found between aging and increased concentrations of IL-10, IL-12(p70), IL-13, IFN-γ, CXCL9, CCL3 and VEGF. The strength of correlation between aging and CCL4 were fair ($r=0.398$). Significant correlation between aging and increased concentrations of IL-1β, IL-4 and IL-5 were also found. However, it should be noted

Table 2 Aqueous cytokine concentrations of child and adult cataract patients

Cytokines	Child			Adult			[a]*P*	LOD
	Mean±SEM	Median	Range	Mean±SEM	Median	Range		
IL-1β	0.08±0.01	0.1	0.02-0.1	0.1±0.0	0.1	0.02-0.2	0.188	0.6
TNF-α	3.7±0.5	3.7	0.9-6.5	2.6±0.2	2.4	0.01-6.7	0.065	6.0
IL-2	0.8±0.2	0.3	0.06-2.5	0.3±0.1	0.1	0.06-3.4	0.099	1.6
IFN-γ	5.9±0.7	6.3	2.3-9.3	8.9±0.8	8.2	0.5-53.62	0.039	6.4
IL-4	0.2±0.05	0.2	0.03-0.4	0.1±0.01	0.07	0.0-0.43	0.206	0.7
IL-5	0.1±0.03	0.2	0.0-0.3	0.3±0.0	0.3	0.0-0.61	0.059	0.5
IL-13	1.7±0.3	1.4	0.7-4.5	3.5±0.4	3.3	0.2-15.54	0.003	0.7
IL-6	5.6±3.4	2.4	0.6-43.7	36.6±14.0	7.7	1.0-556.9	0.001	2.6
IL-12(p70)	3.7±0.9	2.8	0.7-11.6	13.8±0.9	14.0	0.1-27.5	0.000	3.5
IL-10	1.3±0.2	1.0	0.5-3.01	3.2±0.2	3.1	0.1-6.8	0.001	0.3
IL-1Ra	3.4±0.7	3.7	0.04-8.5	2.4±0.4	1.7	0.04-13.9	0.188	5.5
IL-17	5.5±1.5	4.2	0.02-13.7	1.3±0.3	0.9	0.02-18.4	0.049	3.3
CCL2	119.3±13.9	113.3	69.9-246.7	208.3±17.5	171.0	94.4-1305.5	0.000	1.1
CCL3	0.3±0.05	0.4	0.1-0.6	0.7±0.1	0.6	0.0-2.82	0.013	1.6
CCL4	5.5±0.7	4.6	2.4-9.8	19.6±1.6	14.9	4.9-49.9	0.000	2.4
CCL5	1.3±0.3	1.0	0.01-4.2	3.4±1.0	1.2	0.01-44.7	0.343	1.8
CXCL8	1.8±0.6	1.3	0.5-8.4	11.37±2.7	6.7	1.08-166.9	0.000	1.0
CXCL9	29.0±5.1	23.2	17.5-50.4	176.6±23.7	99.3	27.0-980.0	0.000	1.2
CXCL10	130.5±49.4	75.7	18.0-632.4	226.9±24.6	168.2	70.7-1036.1	0.005	6.1
CCL11	0.03±0.1	0.0	0.02-0.18	0.4±0.1	0.0	0.02-6.1	0.241	2.5
VEGF	26.7±5.8	20.2	9.8-84.2	52.8±3.5	50.5	0.4-156.4	0.001	3.1
IFN-α2	21.8±0.8	25.4	18.8-24.8	39.8±1.2	39.1	24.7-59.5	0.000	4.3

Cytokine concentrations were in the unit of pg/mL. SEM: Standard error of mean. LOD: Limit of detection as given by Bio-Rad for individual cytokines in the unit of pg/mL. [a]*P* value between the two groups by Mann-Whitney *U* test.

Table 3 Correlation between age and individual cytokine

Cytokines	*r*	95% CI	*P*	*n*
IL-10	0.568	0.3465-0.7264	<0.0001	71
IL-12(p70)	0.614	0.3764-0.7423	<0.0001	71
IL-13	0.503	0.407-0.7581	<0.0001	71
VEGF	0.594	0.3888-0.7487	<0.0001	71
IFN-γ	0.513	0.3539-0.7304	<0.0001	71
CXCL9	0.532	0.2011-0.7194	0.0017	56
CCL3	0.536	0.2542-0.6749	0.0001	71
CCL4	0.398	0.1406-0.6051	0.0026	71
IL-1β	0.359	0.09598-0.5757	0.0071	71
IL-4	0.305	0.0354-0.5335	0.0235	71
IL-5	0.530	0.301-0.7015	<0.0001	71

r: Correlation coefficient; CI: Confidence interval; *n*: Number of aqueous samples analyzed.

that these three cytokines were at very low concentrations in aqueous. Scatter graphs of cytokine concentration and age for representative cytokines were shown in Figure 1.

DISCUSSION

Cataract is one of the major age-related ocular disorder. Extensive research showed that metabolic changes of the lens is the major causes of cataract. Unlike other age related ocular conditions, such as glaucoma, age-related macular degeneration and diabetic retinopathy which are all featured by some degrees of chronic non-resolving inflammation, cataract is generally believed to be a non-inflammatory condition. For this reason, aqueous taken from adult cataract patients are often used as control to illustrate inflammatory changes associated with other ocular conditions, including inflammatory conditions of the retina[8-11]. However, little is known about the immunological changes associated with age and cataract in adults. In this study, we measured representative Th1, Th2 and innate immunity related inflammatory cytokines and chemokines in aqueous samples of adult and young child cataract patients. Compared to aqueous from young children, the adult aqueous showed increased concentrations of 13 cytokines: IFN-γ, IL-6, IL-13, IL-12(p70), IL-10, IFN-α2, CCL2, CCL3, CCL4, CXCL8, CXCL9, CXCL10 and VEGF. We also identified several cytokines which showed significant age-associated change in the aqueous. Collectively, these cytokines changes suggested an immune milieu in adult aqueous which is pro-angiogenic and pro-inflammatory.

Most of the cytokines listed above are suggestive of a pro-inflammatory change and were similar to that in the aging retina. For example, increased concentrations of CCL2, IL-6 and IL-10 were also found in retina of old age[12-14]. Aging retina is associated with parainflammation, which was featured by increased activity of resident macrophage[15]. While none

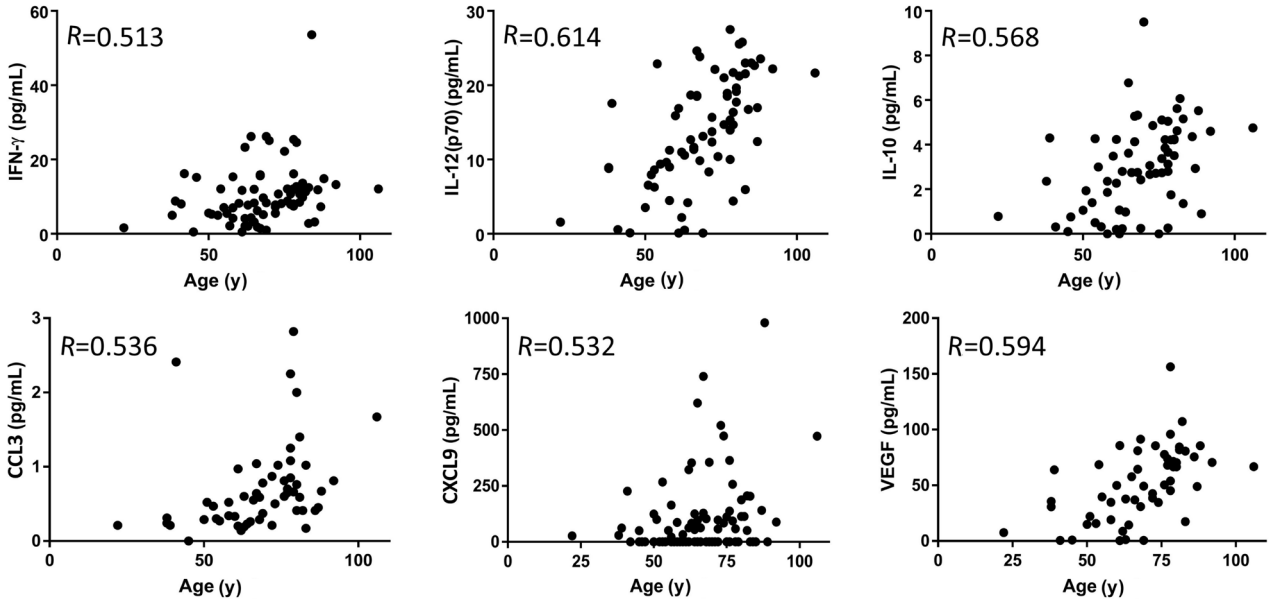

Figure 1 Correlation between age and individual cytokine by Pearson binary correlation analysis controlled for both axial length and the duration of the cataract.

of the aqueous cytokines is produced by a single cell type within the eye, we believe that the increased levels of CCL2, IL-6, IL-12(p70), CCL3 and CCL4 suggested the activation of resident macrophage and the elevated levels of IFN-γ, CXCL9, CXCL10, but not IL-2, IL-4 or IL-5 suggested the activation of natural killer cells. The increased IL-10 concentration suggested a balanced change of the immunological milieu in the adult aqueous. It should be noticed that despite the increase of pro-inflammatory cytokines, the overall profile did not suggest active inflammation since the concentrations of IL-1β and TNF-α remained unchanged and at very low level between young cataract aqueous and adult aqueous.

Our study also revealed a significant age-associated increase of VEGF in the aqueous, indicating a pro-angiogenic change with aging. High level of VEGF is associated with the development of retinal neovascularization. In the eye, the pigmented epithelial cell is a major cellular source for VEGF production. Stress and senescence are major driving forces in simulating the expression of VEGF in cells such as RPE and Müller cells[16-19]. However, unlike the posterior part of the eye, the anterior segment rarely has conditions associated with uncontrolled vascularization. Although VEGF is often associated with angiogenesis, it is also an important survival signal for both vascular and nonvascular tissues. A recent study showed that VEGF also participate in the development of cataract[20]. Therefore the role of elevated VEGF in the aqueous is complicated.

The concentration of IL-6 increased about 6-fold in aqueous humor from child to adult. However, it did not exhibit an age-related change in the adult aqueous. The elevation of IL-6 concentration is an important indicator of intracellular inflammation. Increased concentration of IL-6 was found associated with uveitis[8,21-23], glaucoma[10,24], diabetic retinopathy, age-related macular degeneration and others[9,25-28]. It is likely also an indicator of parainflammation in the aged aqueous humor. A further elevation of its concentration might indicate the disruption of parainflammation in the aqueous.

This study has several limitations. One of the major one is that the aqueous samples were taken from those with congenital cataract. The pathogenesis of congenital cataract is different from that of adult cataract and how it affects aqueous cytokine concentration is unclear. Similarly, it is likely that the aqueous profiles in the adults also related to the development of cataract. Therefore the changes we observed here reflected the combined effect of age and cataract. To minimize the possible another limitation was the number of samples in the adult group. Naturally most of the cataract patients were at the age of 70y and above. To study age-related changes in cytokines, especially the effect of aging on aqueous cytokines, more samples with a wider age range would give more reliable results.

In summary, this study delineated an age-related, overall pro-inflammatory and pro-angiogenetic changes in the cataract aqueous, most likely depicted a parainflammatory status of the aqueous humor similar to what was observed in aging retina. Age-associated changes reflected the combined effect of chronic stress, and it ultimately lead to the shift of the local homeostatic set point. Although the anterior segment does not have as many age-related diseases as the retina, the changes of the cytokines in the aging aqueous may affect the immunological responses in the presence of infection and tissue damage.

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