Effect of Soluble Inducible Costimulator Level and Its Polymorphisms on Age-Related Macular Degeneration

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Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly population. Evidence has shown that the human immune system may play critical roles in this disease. Inducible costimulator (ICOS) promotes T-cell activation, differentiation, and T:B-cell interactions. The aim of the study was to understand the effect of ICOS on the development of AMD from genetic polymorphism perspective and serum level perspective. Two ICOS polymorphisms, rs10183087A/C and rs10932037C/T, were tested in 223 AMD cases and 262 healthy controls. The serum level of soluble ICOS (sICOS) was compared among subjects with different genotypes, as well as between AMD patients and controls. Data showed that prevalence of rs10183087CC genotype was significantly increased in AMD than in controls ($p=0.001$). Function analysis revealed that subjects carrying rs10183087CC genotype had higher serum levels of sICOS than those with AA or AC genotypes ($p<0.05$). When we compared serum levels of sICOS between cases and controls, results showed that AMD patients had significantly increased sICOS levels than healthy donors ($p<0.05$). Also, wet type cases were observed to have higher sICOS levels than cases with dry type ($p<0.05$). These data suggested ICOS polymorphism could affect the susceptibility to AMD by elevating protein expression, and serum levels of sICOS may be closely correlated with the development and progression of this disease.

Introduction

AGE-RELATED MACULAR DEGENERATION (AMD) is currently the leading cause of blindness in many countries and its prevalence will continue to increase in the coming decades unless new prevention strategies can be developed (Meyer *et al.*, 2010). Currently, over 1.75 million U.S. citizens are affected with advanced AMD, and this number is likely to increase to nearly 3 million by the year 2020 due to the increased number of aging U.S. citizens (Meyer *et al.*, 2010). The mechanisms leading to the development of both the early and late lesions of AMD remain largely unknown. Population studies have helped to determine risk factors for this disorder. Some of the nonmodifiable factors include age, genetic factors, light colored iris, and hyperopia (Coleman *et al.*, 2008; Jager *et al.*, 2008). Some of the modifiable risk factors include cigarette smoking, diet, high blood pressure, and possibly elevated serum cholesterol. Recent information supports the notion that immune mechanisms play an important and perhaps central role in AMD (Coleman *et al.*, 2008; Jager *et al.*, 2008).

Inducible costimulator (ICOS) is the third member of the CD28 family of costimulatory molecules, and is induced on

the cell surface following T-cell activation (Swallow *et al.*, 1999; Greenwald *et al.*, 2005). It has been reported that the ICOS pathway promotes T-cell activation, differentiation and effector responses, and T-cell-dependent B-cell responses (McAdam *et al.*, 2000; Coyle and Gutierrez-Ramos, 2004; Nurieva *et al.*, 2008). ICOS mediated costimulation of T cells leads predominantly to the production of effector cytokines such as interleukin (IL)-4 and IL-10, and to a lesser extent, IL-2, interferon (IFN)- γ , and TNF- α (Lohning *et al.*, 1998), thereby playing a more important role in Th2 responses than Th1 responses (Burmeister *et al.*, 2008; Bauquet *et al.*, 2009). Due to the importance of ICOS, aberrant ICOS expression has been shown to be associated with different diseases. ICOS surface expression has been found to be increased on T cells from patients with rheumatoid arthritis, and ligation of ICOS induces augmented IFN- γ production (Okamoto *et al.*, 2003). Patients with systemic lupus erythematosus were also found to have increased ICOS expression on their peripheral blood T cells (Hutloff *et al.*, 2004).

ICOS may play critical roles in AMD. ICOS upregulates IL-10 and INF- γ , those have profound effects on AMD (Rudd *et al.*, 2009). More importantly, ICOS can activate complement cascade, which is one of the main causes for the

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development of AMD (Jain *et al.*, 2008; Sharpe, 2009). In the current study, we have investigated the role of ICOS on AMD from two different angles. First of all, we have tested ICOS rs10183087A/C and rs10932037C/T polymorphisms in AMD and analyzed their functions. Second, we have analyzed the serum levels of soluble ICOS (sICOS) in this disease.

Materials and Methods

Patients and controls

The study group included 223 AMD cases and 262 healthy controls recruited from the General Hospital of Guangzhou Military Command of PLA. The inclusion criteria for patients included those with age 50 years or older with the diagnosis of AMD. AMD was defined by geographic atrophy and/or choroidal neovascularization with drusen more than five in at least one eye. The exclusion criteria included the retinal diseases involving the photoreceptors and/or outer retinal layers other than AMD loss such as high myopia, retinal dystrophies, central serious retinopathy, vein occlusion, uveitis, or similar outer retinal diseases that have been present before the age of 50 and opacities of the ocular media, limitations of papillary dilation, or other problems sufficient to preclude adequate stereo fundus photography. The control group was recruited from people who underwent regular physical examinations at the same hospital. The study was approved by the Review Board of the General Hospital of Guangzhou Military Command of PLA. Each study participant provided a peripheral blood sample.

DNA extraction and genotyping

Genomic DNA was extracted from 5 mL frozen whole blood using the DNA Extraction Kit (Fastagen) according to the manufacturer's protocol. The ICOS rs10183087A/C and rs10932037C/T genotypes were determined using a polymerase chain reaction (PCR)–restriction fragment length polymorphism assay and DNA sequencing analysis. The PCR primers for the rs10183087A/C and rs10932037C/T polymorphisms were 5'-AGAGGGGET AL.,GCTTCTTG TAGGA-3' (forward) and 5'-CTCATCCCTACAGET AL., GAAGAGGT-3' (reverse), and 5'- CATTATCTATGTTTTC ATGGTGCTATT-3' (forward) and 5'- AGGCTATCTTGAA GGGCCAG-3' (reverse), respectively. The PCRs were performed in a total volume of 25 μ L containing 100 ng genomic DNA, 20 pM of each primer, 0.2 mM dNTPs, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, and 1 unit of Taq polymerase (New England BioLabs). The PCR cycle conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30 s at 94°C; 45 s at 61°C for rs10183087A/C and 63°C for rs10932037C/T; 45 s at 72°C; and a final elongation at 72°C for 8 min. The PCR products were digested for 3 h at 37°C with the appropriate restriction enzymes (New England BioLabs). The restriction enzymes for the rs10183087A/C and rs10932037C/T genotypes were *Hpy*188III and *Hsp*92II, respectively. The digested PCR products were resolved on 8% acrylamide gels and stained with ethidium bromide for visualization under UV light. As for the rs10183087, the fragment size of PCR was 532 bp. When conducting enzyme digestion, the fragment size indicating the A allele was 223 and 309, whereas the C allele was 90,

133, and 309. As for rs10932037, the fragment size of PCR was 445 bp. When conducting enzyme digestion, the fragment size indicating the C allele was 19, 76, 80, and 270, whereas the T allele was 19, 156, and 270. To confirm the genotyping results, more than 10% of PCR amplified DNA samples were examined by DNA sequencing. Results between PCR and DNA sequencing analysis were 100% concordant.

Measurement of serum sICOS levels

Serum levels of sICOS were measured in duplicate with a specific sandwich ELISA kit (Usn Life Science, Inc.), according to the manufacturer's protocol.

Statistical analysis

Genotype and allele frequencies of ICOS polymorphisms were compared between AMD cases and controls using the chi-square test and odds ratios (OR), and 95% confidence intervals (CIs) were calculated to assess the relative risk conferred by a particular allele and genotype. Demographic and clinical data between groups were compared by the chi-square test and Student's *t*-test. Haplotypes of the SNPs were conducted and analyzed using the SHesis software (Bio-X, Inc.). The serum level of sICOS was compared by the Student's *t*-test. Statistical significance was assumed at $p < 0.05$. The SPSS statistical software package version 13.0 was used for all of the statistical analyses.

Results

Clinical characteristics of the study subjects

The clinical characteristics of all the subjects are shown in Table 1. There was no significant difference in age ($p > 0.05$) and gender ($p > 0.05$) between the AMD patients and the healthy adults. Compared to the control, the AMD group had higher levels of body mass index (BMI) (24.8 ± 6.9), and higher prevalence of hypertension (18.8%) as well as diabetes (9.4%). However, none of these values showed significant differences. In the 223 AMD cases, 152 (68.2%) were wet type, whereas 71 (31.8%) were dry type.

The ICOS polymorphisms in AMD cases and controls

The genotype and allele frequencies of the ICOS rs10183087A/C and rs10932037C/T polymorphisms in

TABLE 1. GENERAL CHARACTERISTICS OF THE AGE-RELATED MACULAR DEGENERATION PATIENTS AND CONTROL GROUP

Characteristics	AMD (n = 223)	Control (n = 262)	p-Value
Age (years)	66.7 $\pm$ 8.2	68.3 $\pm$ 7.6	>0.05
Gender (M/F)	125/98	146/116	>0.05
BMI (kg/m ²)	24.8 $\pm$ 6.9	23.3 $\pm$ 4.9	>0.05
Hypertension, n (%)	42 (18.8)	38 (14.5)	>0.05
Diabetes, n (%)	21 (9.4)	17 (6.5)	>0.05
Wet AMD	152 (68.2)	—	
Dry AMD	71 (31.8)	—	

Data are presented as mean $\pm$ standard deviation.

AMD, age-related macular degeneration; M/F, male/female; BMI, body mass index.

AMD cases and controls are summarized in Table 2. The genotype distributions of these two polymorphisms among the controls were in agreement with the Hardy-Weinberg equilibrium ($p>0.05$). The allele frequencies of ICOS rs10183087A/C polymorphism were 77.1% and 22.9% among the cases, and 86.3% and 13.7% among the controls ($p=0.0002$). Prevalence of the ICOS rs10183087CC genotype was significantly higher in patients than in controls (OR=3.81, 95% CI: 1.65–8.80, $p=0.001$). As for the rs10932037C/T polymorphism, the prevalence of T and G allele was 96.4% and 3.6% in patients and 97.1% and 2.9% in controls ($p=0.522$). We did not identify the ICOS rs10932037TT genotype in controls. The ICOS rs10932037CT genotype did not show any significant difference between the patients and the controls (OR=1.11, 95% CI: 0.52–2.35, $p=0.788$). In addition, we analyzed the haplotypes of the two polymorphisms. No significant linkage disequilibrium was observed among these SNPs. Haplotypes are shown (Table 2). The CC (rs10183087–rs10932037) haplotype had significantly higher numbers in AMD cases compared to healthy controls (OR=1.84, 95% CI: 1.31–2.59, $P=0.0004$). These data suggested that the ICOS rs10183087A/C polymorphism was associated with an increased susceptibility to AMD in the Chinese population.

Comparison of ICOS polymorphisms on protein expression

To learn the mechanism of the association between ICOS polymorphisms and AMD, we first compared the effect of rs10183087A/C polymorphism on the serum level of sICOS in healthy controls (Fig. 1A). To avoid the potential inter-

TABLE 2. ICOS POLYMORPHISMS IN AGE-RELATED MACULAR DEGENERATION PATIENTS AND CONTROLS

Polymorphism	Cases n=223 (%)	Controls n=262 (%)	OR (95%CI)	p-Value
rs10183087				
Genotype				
AA	143 (64.1)	198 (75.6)	1.00	
AC	58 (26.0)	56 (21.4)	1.43 (0.94–2.20)	0.096
CC	22 (9.9)	8 (3.0)	3.81 (1.65–8.80)	0.001 ^a
Allele				
A	344 (77.1)	452 (86.3)	1.00	
C	102 (22.9)	72 (13.7)	1.86 (1.34–2.60)	0.0002 ^a
rs10932037				
Genotype				
CC	208 (93.3)	247 (94.3)	1.00	
CT	14 (6.3)	15 (5.7)	1.11 (0.52–2.35)	0.788
TT	1 (0.4)	0 (0.0)	—	—
Allele				
C	430 (96.4)	509 (97.1)	1.00	
T	16 (3.6)	15 (2.9)	1.26 (0.62–2.58)	0.522
Haplotypes				
rs10183087–rs10932037				
AC	335 (75.1)	441 (84.2)	1.00	
CC	95 (21.3)	68 (13.0)	1.84 (1.31–2.59)	0.0004 ^a
AT	9 (2.0)	11 (2.1)	1.08 (0.44–2.63)	0.870
CT	7 (1.6)	4 (0.7)	2.30 (0.67–7.94)	0.174

^ap-Value is statistically significant (<0.05).

ICOS, inducible costimulator; OR, odds ratio; CI, confidence interval.

FIG. 1. Serum level of soluble inducible costimulator (sICOS) in the study population with different polymorphisms. (A) Shown in healthy controls. (B) Shown in age-related macular degeneration (AMD) patients. Data are presented as mean $\pm$ standard deviation (SD).

ference from rs10932037C/T polymorphism, healthy donors carrying this genetic variant were excluded. Data showed that the serum level of subjects with wild-type rs10183087AA genotype was 466 ± 98 pg/mL (mean $\pm$ standard deviation), whereas subjects with AC and CC genotypes were 496 ± 107 pg/mL and 704 ± 108 pg/mL, respectively. Subjects with rs10183087CC genotype had a significantly higher level of sICOS than those with wild-type AA and AC genotypes ($p<0.05$) (Fig. 1A). We also compared the effect of rs10932037C/T polymorphism on the serum level of sICOS in healthy controls (Fig. 1A). Similarly, subjects having rs10183087A/C polymorphism were excluded to avoid interference. Results revealed that serum levels of subjects with rs10932037CC and rs10932037CT genotypes were 449 ± 91 pg/mL and 478 ± 111 pg/mL, respectively. No significant differences were observed between CC and CT genotypes (Fig. 1A). By using similar methods, we further compared the effect of these two polymorphisms on the serum level of sICOS in AMD patients (Fig. 1B). Patients with rs10183087CC genotype demonstrated elevated levels of sICOS than patients with AA and AC genotypes ($p<0.05$), whereas rs10932037C/T polymorphism did not present any effect on the sICOS levels in AMD patients (Fig. 1B). These results indicated that the rs10183087A/C polymorphism could increase the sICOS serum level in both healthy subjects and AMD cases.

FIG. 2. Comparison of serum sICOS between controls and AMD patients (A). Comparison of serum sICOS levels between AMD cases with hypertension and AMD cases without hypertension (B); between AMD cases with diabetes and AMD cases without diabetes (C); between cases with wet type and cases with dry type (D). Data are presented as mean $\pm$ SD.

Serum level of sICOS in AMD cases and controls

To understand the effect of ICOS on AMD, we compared the serum level of sICOS in AMD cases and controls (Fig. 2A). Subjects with ICOS polymorphisms were excluded to avoid interference. As shown in Figure 2A, an increased sICOS expression was observed in AMD patients (597 ± 99 pg/mL) than in healthy controls (466 ± 98 pg/mL) ($p < 0.05$). To learn whether the ICOS level is correlated with some clinical parameters, we analyzed serum ICOS in patients with different hypertension status (Fig. 2B), diabetes status (Fig. 2C), or vessel affected status (Fig. 2D). Data showed that the serum ICOS level was not associated with hypertension or diabetes in AMD ($p > 0.05$). However, AMD patients who had wet type presented significantly higher sICOS levels than those with dry type (700 ± 150 pg/mL versus 494 ± 56 pg/mL, $p < 0.05$) (Fig. 2D). These results indicated that serum sICOS may play roles in the progression of AMD.

Discussion

Evidence is accumulating that the human immune system is closely correlated with the development of AMD (Coleman *et al.*, 2008; Jager *et al.*, 2008). However, factors responsible for the pathogenesis of AMD remain unclear. The current study investigated the effect of ICOS on AMD from various angles. We first identified that ICOS rs10183087A/C polymorphism could affect the susceptibility to AMD by increasing protein expression. Further, we found that the serum sICOS level was elevated in AMD patients and associated with wet type. This study demonstrated that ICOS may play critical roles in the development and progression of AMD.

ICOS rs10183087A/C and rs10932037C/T SNPs were located at the 3' untranslated region (3'UTR) (Haimila *et al.*, 2002), by which the gene expression and mRNA stability may be influenced (Wu *et al.*, 2009). Studies have presented

that polymorphisms of ICOS may be associated with various diseases. It has been reported that recipients of hematopoietic stem cell transplantation who received a graft from a donor with ICOS rs10183087CC genotype had worse disease-free survival (Wu *et al.*, 2009). In addition, Castelli *et al.* (2007) investigated eight SNPs at the ICOS 3'UTR and mentioned that the homozygote of haplotype A (602A-930G-1459A-1564T-1624C-1862A-2007G-2373G) decreased ICOS expression and might play a protective role in multiple sclerosis. Our data have shown that the ICOS rs10183087CC genotype can significantly increase the serum level of sICOS in healthy controls (Fig. 1A), suggesting a possible function of this polymorphism in gene expression. Similarly, AMD patients with the ICOS rs10183087CC genotype also showed higher sICOS levels, indicating that the effect of ICOS rs10183087A/C polymorphism on protein expression is ubiquitous, or at least is not restricted in healthy people. As for the rs10932037C/T SNP, previous research has reported that recipients of hematopoietic stem cell transplantation with the ICOS rs10932037TT genotype had an overall worse survival. Our study did not observe any changes in sICOS levels with regard to the polymorphism (Fig. 1), suggesting that it may not affect the gene expression at the protein level.

Increased ICOS expression has been associated with disease in several human studies. ICOS surface expression has been found to be increased on T cells from patients with rheumatoid arthritis, and ligation of ICOS induces augmented INF- γ production (Okamoto *et al.*, 2003). Patients with systemic lupus erythematosus were also found to have increased ICOS expression on their peripheral blood T cells (Hutloff *et al.*, 2004). The increase in ICOS expression correlated with increased IL-4, IL-5, and IL-13 production from *ex vivo* stimulated peripheral blood mononuclear cells. In addition, studies have suggested that the level of ICOS expression may contribute to the proliferation and migration of Tfh (Shilling *et al.*, 2006). In the current study, we observed

elevated sICOS levels in AMD patients, especially in patients with wet type. The mechanism remains unclear yet. Since ICOS regulates multiple cytokine productions, whereas cytokines may play critical roles in the development of AMD, we hypothesize that ICOS may affect AMD indirectly through cytokines. In addition, we identified that AMD with wet type had higher sICOS levels than those with dry type (Fig. 2D), suggesting that sICOS may be closely involved in the development of the disease. Further studies on this field are necessary.

In summary, our study identified that ICOS polymorphism could affect the susceptibility to AMD by increasing ICOS expression, and found that the serum sICOS level was elevated in AMD patients, especially in patients with wet type. This research sheds light on understanding the pathogenesis of AMD and provides therapeutic strategies in this disease.

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Disclosure Statement

No competing financial interests exist.

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