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The involvement of sequence variation and expression of CX3CR1 in the pathogenesis of age-related macular degeneration

JINGSHENG TUO^{*}, BRENA C. SMITH^{*}, CHRISTINE M. BOJANOWSKI^{*}, ANNAL D. MELETH^{*,†},
IGAL GERY^{*}, KARL G. CSAKY[§], EMILY Y. CHEW[†], and CHI-CHAO CHAN^{*,2}

^{*}Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA

[†]Division of Epidemiology and Clinical Research, National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA

[§]Section of Gene Therapy, National Eye Institute, National Institutes of Health, Bethesda, Maryland, USA

SPECIFIC AIM

There is a significant genetic component associated with the risk of developing age-related macular degeneration (AMD), a leading cause of blindness among older people in Western countries. Based on the recognized role of CX3CR1, a chemokine receptor, in other age-related complex diseases and the possible involvement of the immune system in AMD pathogenesis, we tested for an association between *CX3CR1* sequence variation/expression and AMD by screening two *CX3CR1* single nucleotide polymorphisms (SNPs), V249I and T280M, among AMD patients and controls, and determining the level of CX3CR1 expression in AMD and normal eye tissues.

PRINCIPAL FINDINGS

1. An association between *CX3CR1* 1249/M280 alleles and AMD

A significant increased prevalence of M280 and 1249 carriers or alleles was found among AMD cases vs. controls. Distribution of *CX3CR1*V249I and T280M did not deviate from Hardy-Weinberg equilibrium in any group (Table 1).

2. A quantitative correlation between *CX3CR1* 1249/M280 and the prevalence of AMD phenotype

As indicated in Table 1, the odds ratios (OR) of clinically diagnosed AMD compared with controls were 1.86 and 1.94 in terms of carrying 1249 and M280, respectively; however, the OR of pathologically diagnosed AMD reached 3.57 in terms of carrying M280. A higher OR was found for the clinically diagnosed AMD group compared with the aged controls whereas a lower OR was observed for the AMD group compared with the blood donor population of a much younger age. Allele frequency analysis yielded similar results (see full text version).

3. Lower expression of *CX3CR1* in the AMD maculae from individual bearing *CX3CR1* T/M280 compared with individual bearing *CX3CR1* T/T280

CX3CR1 transcripts were undetectable in the maculae of AMD individuals bearing T/M280. However, *CX3CR1* transcripts were detected in normal macula of the T/T280 or T/M280 individual and AMD macula from the individual bearing T/T280. Normal T/T280 macular

²Correspondence: 10 Center Dr., Bldg. 10, Rm. 10N103, NIH/NEI, Bethesda, MD 20892-1857, USA. E-mail: chanc@nei.nih.gov

[†]HHMI-NIH Research Scholar.

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cells expressed the highest level of *CX3CR1* mRNA; AMD T/T280 macular cells exhibited the lowest level. The level of *CX3CR1* mRNA expression in the normal macular cells bearing T/M280 fell between these two values (Fig. 1). *CX3CR1* protein, as detected by immunohistochemical staining, exhibited a similar pattern of expression, with less *CX3CR1* intensity stained in the RPE and adjacent tissues in the AMD section bearing T/M280 compared with the intensity stained from both normal and AMD maculae bearing the T/T280.

CONCLUSIONS AND SIGNIFICANCE

Our results suggest that *CX3CR1* 1249 and M280 are associated with an enhanced risk of AMD development. Statistically significant differences in frequencies of both SNPs were observed between the AMD case group and controls. These findings have not been reported before in the literature.

We also compared expression of *CX3CR1* mRNA and protein in AMD macular cells obtained from archived paraffin-embedded tissue sections. *CX3CR1* transcripts and protein in the AMD maculae were undetectable or expressed at much lower levels than their SNP-type counterparts in the normal eyes. In normal eyes, macular cells from the T/M280 individual exhibited lower *CX3CR1* transcript expression than the individual bearing T/T280. We found lowered *CX3CR1* expression in the macula compared with the perimacular retina of AMD eyes bearing T/M280. In contrast, similar levels of *CX3CR1* expression were detected in macular and perimacular regions of the normal subjects (data not shown). These observations suggest that reduced *CX3CR1* expression in the macular area may also contribute to AMD development.

The results of our multiple case control design strengthened the conclusion that there is an association between *CX3CR1* sequence variation and AMD. The higher OR found in the pathologically diagnosed AMD seems logical, as pathological diagnoses are more accurate and definitive than clinical diagnoses. This result suggests that clearly defined phenotypes will increase the likelihood of successful risk factor discrimination. Although difficulty was met in recruiting age-matched controls, the control group obtained with a younger average age does not increase the likelihood of a false positive. It is highly possible that some individuals in the control group with a younger mean age will develop AMD later in life due to the high incidence and age-related nature of the disease. If *CX3CR1* polymorphisms are truly risk factors for AMD, then an even greater difference in the polymorphic allele frequencies between the case and control groups is anticipated if an age-matched control population is included. Consistent with this, a lower OR was observed for the AMD group than the blood donor group, which had a much lower mean age.

The association of the *CX3CR1* SNPs with various cardiovascular diseases has been reported. Studies have indicated a protective role for 1249 and M280 against cardiovascular diseases with an OR of 0.43. The mechanism underlying this protective effect observed in the cardiovascular diseases may involve reduced activities of the chemokine system caused by a lowered affinity and decreased number of binding sites of *CX3CR1*. A retarded chemokine system may in turn limit the macrophage-induced/triggered plaque formations that serve as the hallmark of these cardiovascular diseases. Although several pathological features are shared between AMD drusen and arteriosclerosis plaques, the findings of epidemiological studies on the association between AMD and hypertension remain controversial.

Extending the recent AMD animal model findings as to the role of chemokines, we propose a model that may account for the association of AMD with the *CX3CR1*1249/M280 sequence variants (Fig. 2). Under normal conditions, a dynamic balance is struck between the generation of macular deposition stimulated by internal and external factors and the elimination of these deposits by inflammatory cells attracted to the site by chemokines. In this model, *CX3CR1*

sequence variation results in a decreased number of binding sites and diminished binding affinity, crippling chemokine activity. Decreased chemokine function may lead to inadequate recruitment of inflammatory cells to the macula, where the age-related deposit is progressively accumulated.

Lower CX3CR1 expression in cells from the degenerative maculae suggests that CX3CR1 levels may play a role in the pathogenesis of AMD. Although associations between I249/M280 and AMD exist, it seems unlikely that these variants affect the level of transcription directly. Instead, the determinants may lie in the regulatory sites of *CX3CR1*, possibly in linkage disequilibrium with V249I and T280M. A recent study reported the linkage disequilibrium of V249I and T280M with three SNPs in the *CX3CR1* promoter region. Of the three SNPs in the promoter region, one was reported to be in the strongest linkage disequilibrium with both V249I and T280M in the Caucasian population.

Several SNPs of various other candidate genes have been analyzed for AMD association using the same AMD case and control samples in this study. Statistically, a lower *P* value is required to assume significance so as to reduce the likelihood of obtaining a false positive for a single SNP among the multiple SNPs being tested. Larger sample population groups are required to further confirm the association observed presently. Repeat studies of other ethnic populations would further strengthen our conclusions.

The allele frequency of M280 varies from 13.5% to 20.2% in general populations as reported in other association studies. According to LocusLink, the average heterozygosity of M280 based on all submitted variation data are 11.2%, a frequency similar to the level reported in our control group.

A chronic inflammation theory has been proposed to describe the possible etiology of macular drusen formation. Local inflammation is now recognized as a tandem element of numerous chronic diseases characterized by the progressive accumulation of insoluble deposits. Analyses of the drusen components from AMD patient autopsy samples have led to the identification of many inflammatory mediators, such as activated complement components and acute phase proteins. The cumulative deposit from chronic tissue-specific inflammation, if not efficiently eliminated by relevant factors such as proper macrophage and chemokine system function, significantly exacerbates the effects of the primary pathogenic lesions. However, macrophages may play a different role in the retina, where their function is to repair the damage of acute inflammation. A typical process of acute inflammation is induced experimentally in mice by laser treatment followed by intense infiltration of inflammatory cells, mainly macrophages. An adverse consequence of this process is the development of neovascularization, a process attributed to macrophage activity. Indeed, this notion is supported by the observation that systematic macrophage depletion reduces the level of neovascularization after laser treatment in mouse eyes.

CX3CR1 1249 and M280 account for ~10% of the total risk in our study group, the difference of allele frequency between AMD and control populations. Current evidence reveals it is highly unlikely that a single gene variant is solely responsible for the development of AMD, at least not in the common forms of the disease. Larger sample sizes are needed to adequately study gene-gene and gene-environmental interactions and determine additional AMD risk factors.

Since AMD is an age-dependent disease of high incidence, multiple control groups of different average ages can be used to elucidate a possible dose-response of the genetic risk tested. We attempted such an approach using a study design that compensates for our small sample sizes in order to enhance the power of this study. Only advanced AMD cases were included to minimize a possible bias introduced by poorly classified phenotyping criteria. Continuous

studies in a cohort with quantitative disease scoring will be invaluable in the attempt to discriminate the genetic component of AMD.

In conclusion, this study demonstrates that *CX3CR1* 1249 and M280 alleles, as well as CX3CR1 expression in the macular cells, may be associated with an increased risk of AMD development. We propose that one of the underlying mechanisms of AMD development involves a decrease in the chemoattractant efficiency of inflammatory cells to the retina of individuals exposed to AMD risk factors.

Figure 1.

RT-PCR of *CX3CR1* in maculae obtained from AMD and normal paraffin-embedded slides of eyes with different SNP types. Small amplicons of 100 bp spanning exon 1 and exon 2 of *CX3CR1* and 103 bp spanning from exon 5 to exon 6 of β -actin were amplified using ^{32}P -labeled primers.

Figure 2.

a) Flowchart of proposed model that may account for the association of AMD with the *CX3CR1* I249/M280 sequence variants. *CX3CR1* sequence variation results in a decreased number of binding sites and diminished ligand binding affinity, thus crippling chemokine activity. A decrease in proper chemokine function may lead to the inadequate recruitment and subsequent decrease in activity of macro-phages in macular regions where the age-related deposit is progressively accumulated, b) Schematic diagram showing that in the wild-type a dynamic balance is struck between the generation of macular deposition and elimination of these deposits by inflammatory cells, c) Aberrant macrophage activity in individuals with the T/M280 and V/I249. The macro-phages are not successfully recruited to sites of macular deposit, allowing a buildup of age-accumulated deposition. This buildup contributes to the formation of drusen and destruction of the RPE cells resulting in the development of AMD.

TABLE 1
SNP type distribution of CX3CR1 V249I and T280M among the study groups^a

	N	CX3CR1-249					CX3CR1-280				
		V/V	V/I (%)	I/I (%)	V/I + I/I OR (CI)	X ² /P	T/T	T/M (%)	M/M (%)	T/M + M/M OR (CI)	X ² /P
Control	105	63	39 (37.1)	3 (2.6)			82	23 (21.9)	0		
Blood donors	171	98	65 (38.0)	8 (4.6)			128	41 (24.0)	2 (1.2)		
CD AMD	85	38	42 (49.4)	5 (5.9)	1.86 (1.04–3.31) [#]	4.41/0.04 [#]	55	27 (31.8)	3 (3.5)	1.94 (1.02–3.69) [#]	4.19/0.04 [#]
					1.66 (0.98–2.8) [‡]	3.02/0.06 [‡]				1.62 (0.92–2.85) [‡]	2.87/0.09 [‡]
PD AMD	32 [‡]						16	13 (43.8)	3 (6.3)	3.57 (1.55–8.20) [#]	9.91/0.002 [#]

^aThe subjects in this case control study with multiple control design included sporadic clinically diagnosed patients with advanced AMD (referred to as the CD AMD, age = 79.1 ± 8.9), randomly recruited normal controls (referred to as controls, age = 68.2 ± 7.8), and healthy blood donors (age = 40.1 ± 12.1). All of the subjects were Caucasian. The normal controls were spouses, friends of AMD case subjects, and normal volunteers. Individuals in the control population underwent a clinical eye examination; the blood donor population did not receive clinical evaluation. Archived paraffin-embedded ocular sections from 40 pathologically diagnosed advanced stage AMD patients were used as the DNA and mRNA source for CX3CR1 SNP typing and ocular expression analysis (referred to as the PD AMD).

[#]In comparison with controls

[‡]In comparison with blood donors

[‡]Genotyping was successful in 32 of the 40 tissue sections.