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Detection of CX3CR1 single nucleotide polymorphism and expression on archived eyes with age-related macular degeneration

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Summary. There is a significant genetic component in age-related macular degeneration (AMD). *CX3CR*1, which encodes the fractalkine (chemokine, *CX3CL1*) receptor, has two single nucleotide polymorphisms (SNPs): V249I and T280M. These SNPs are correlated with other aged-related diseases such as atherosclerosis. We have reported an association of *CX3CR1* SNP and AMD. In this study we examined *CX3CR1* SNP frequencies and protein expression on archived sections of AMD and normal eyes. We microdissected non-retinal, peripheral retinal and macular cells from archived slides of eyes of AMD patients and normal subjects. *CX3CR1* SNP typing was conducted by PCR and restriction fragment length polymorphism analysis. *CX3CR1* transcripts from retinal cells were also measured using RT-PCR. CX3CR1 protein expression was evaluated using avidin-biotin complex immunohistochemistry. We successfully extracted DNA from 32/40 AMD cases and 2/2 normal eyes. Among the 32 AMD cases, 18 had neovascular AMD and 14 had non-neovascular AMD. The M280 allele was detected in 19/64 (32 cases x2) with a frequency of 29.7%, which was significantly higher as compared to the frequency in the normal population (11.2%). We detected *CX3CR1* expression in the various retinal cells. *CX3CR1* transcript and protein levels were diminished in the macular lesions. This study successfully analyzed *CX3CR1* SNP and transcript expression in microdissected cells from archived paraffin fixed slides. Our data suggest that the M280 allele, a SNP resulting in aberrant CX3CR1 and CX3CL1 interaction, as well as lowered expression of macular CX3CR1, may contribute to the development of AMD.

Key words: Age-related macular degeneration, Single nuclear polymorphism, CX3CR1, Chemokine, Macrophage

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Introduction

Age-related macular degeneration (AMD) is the leading cause of visual impairment and blindness in the United States and the developed countries among people 65 years and older (Klaver et al., 1998b; la Cour et al., 2002). However, the etiology of AMD is still unknown. The development and pathogenesis of AMD is determined by a complex interaction between environmental factors and genetic backgrounds. In addition to age, epidemiological studies have identified cigarette smoking and diet as risk factors for AMD development (AREDS, 2000; Seddon et al., 2001; Husain et al., 2002; Hyman and Neborsky, 2002). The tendency for familial aggregation in AMD cases, with approximately 20% of AMD patients having a positive family history, suggests a significant genetic component in disease development as well (De Jong et al., 2001).

The potential role of genetic variation in the development of AMD has received increased attention. Gene variation has been reported in association with various other human age-related diseases such as cancer and the cardiovascular diseases (Halushka et al., 1999; Gulcher et al., 2001; Brennan, 2002). A few studies have also demonstrated an association between AMD and various gene polymorphisms (Schmidt et al., 2000). Examples of genes with single nucleotide polymorphisms (SNPs) that have been found in association with AMD include *SOD2*, a manganese superoxide dismustase (MnSOD) isoform (Kimura et al., 2000), *paraoxonase* (Ikeda et al., 2001), and *apolipoprotein E (ApoE)* (Klaver et al., 1998a; Souied et al., 1998; Simonelli et al., 2001).

Macrophage activity has been shown to play a significant role in AMD pathogenesis (Dastgheib and Green, 1994; Espinosa-Heidmann et al., 2003; Forrester, 2003). For this reason, investigators have begun to further explore the potential role of immunological mechanisms in this disease (Ambati et al., 2003b). Chemokines are a group of small, pro-inflammatory molecules first described for their pivotal role in the

mobilization and subsequent activation upon arrival of specific leukocyte subsets to sites of inflammation (Taub and Oppenheim, 1994). Due to their vast functional responsibilities, chemokines have been linked to the pathogeneses of many seemingly unrelated diseases such as HIV infection, cancer, atherosclerosis, and various autoimmune diseases (Howard et al., 1996; Berger et al., 1999; Balkwill, 2003). The *CX3CR1* gene, which encodes a chemokine receptor for fractalkine (CX3CL1), is known to be polymorphic with two non-synonymous SNPs in the open reading frame. These two polymorphisms, involve a valine to isoleucine substitution at position 249 and a threonine to methionine at position 280 respectively. Several investigators have reported associations between these *CX3CR1* polymorphisms and human HIV progression and arteriosclerosis susceptibility (Faure et al., 2000; McDermott et al., 2000, 2003).

Recently we reported an association between the *CX3CR1* T280M and V249I polymorphisms and AMD risk (Tuo et al., 2004a,b). In a small multiple control study, we reported an increased prevalence of the I249 and M280 alleles among AMD cases when compared to the control populations ($p<0.05$). In order to further investigate the involvement of *CX3CR1* in AMD, we evaluated CX3CR1 messenger and protein expression in addition to performing SNP typing assay on archived slides obtained from patients with histopathologically classified neovascular or non-neovascular AMD.

Materials and methods

Cases

Archived and paraffin-embedded slides of 42 autopsied eyes were collected. Forty eyes with a pathological diagnosis of AMD were obtained from the Wilmer Eye Institute (35 cases) and National Eye Institute (5 cases). All eyes were serially sectioned through the macula via the pupillary optic nerve head axis. Only 1-2 slides per case were available from the material collected from the Wilmer Eye Institute; therefore, only SNP analysis could be performed for these 35 cases. For the 5 cases obtained from the National Eye Institute, SNP analysis, immunohistochemistry, and RT-PCR could all be performed.

All slides were stained for hematoxylin and eosin. Two normal autopsied eyes collected from 70 and 78 year-old Caucasians from the National Eye Institute served as controls. The Institutional Review Board of the National Eye Institute approved this study for human subjects.

Microdissection

The cover slips of the hematoxylin-eosin stained slides were removed. The non-retinal (corneal and/or iris), peripheral retinal and macular retinal cells were then carefully microdissected either manually under a light microscope or using the PixCell IIe laser capture microscope (Arturus, Mountain View, CA). Approximately similar numbers of peripheral and macular retinal cells were obtained from each case. The PixCell IIe uses a low power infrared laser to collect selected cells on a membrane located on the cap of a 1.5 ml tube. In manual microdissection, the selected cells were gently scraped, detached and lifted from the slide using a 30-gauge needle (Shen et al., 1998).

Single nucleotide polymorphism (SNP) assay

The non-retinal cells were immediately placed in proteinase K enriched DNA extraction buffer. PCR amplification was performed using the primers containing the polymorphic *CX3CR1* site (sense, 5'-CCG AGG TCC TTC AGG AAA TCT-3' and anti-sense, 3'-GAG TTC CTG AAC CTG ATG CTG A-5'). SNP assay was performed through PCR amplification followed by restriction fragment length polymorphism (RFLP) analysis.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from both peripheral and macular retinal cells of the 5 cases obtained from the NEI in order to perform RT-PCR for *CX3CR1* and *ß-actin* mRNA. The primers used were 5'-CAG ATC CAG AGG TTC CCT TG-3' and 5'-TAA CAG GCC TCA GCC AAA TC-3' for *CX3CR1* and 5'-TAA CAG GCC TCA GCC AAA TC-3' and 5'-ACA TCT GCT GGA AGG TGG AC-3' for *ß-actin*. Microdissected peripheral and macular retinal cells from two normal autopsied eyes served as controls and were assayed in the same fashion.

Immunohistochemistry

The avidin-biotin-complex immunoperoxidase technique was utilized on the unstained, de-paraffinized slides of 5 NEI cases in which macular sections were available. A normal autopsied eye was also stained and used as a control. The primary antibody was rabbit anti-human CX3CR1 polyclonal antibody (Chemicon International, Inc., Temecular, CA) or control rabbit IgG. The secondary antibody was biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). The substrate was avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA), and the chromogen was diaminobenzidine and nickel sulfate. A deparaffinized section from a normal eye served as a positive control for immunostaining against the CX3CR1 antibody. The positive reaction will result in the production of a blue-blackish color.

Results

Histopathology

All 40 cases demonstrated classical AMD lesions in

the macula as defined in the literature (Green and Enger, 1993; Green, 1999). In general, both eyes of the same case presented similar and rather symmetrical lesions. Subretinal choroidal neovascularization and photoreceptor loss with or without disciform scars were found in 23 of the 40 cases. These cases were diagnosed with neovascular AMD. The remaining 17 cases were diagnosed with AMD showing areolar (geographic) atrophy without neovascularization. These cases were characterized by a loss of photoreceptors, alteration or loss of RPE cells, drusen formation, and/or calcification in the macula. In this paper, we refer to these two groups as either with or without neovascular AMD respectively.

Detection of CX3CR1 T280M polymorphism

Genotyping was carried out in 32 of the 40 cases from which DNA could be successfully extracted from the microdissected ocular cells. A mean age of 82.8 years (ranging from 63-96 years old) was obtained for

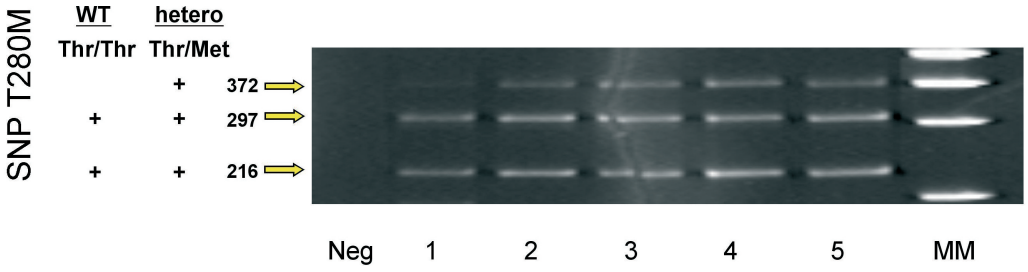

Fig. 1. Autoradiography showing *CX3CR1* SNP typing of 5 AMD cases from the National Eye Institute: one with Thr/Thr (case 1) and 4 with Thr/Met genotypes. The restriction enzyme used to digest the PCR T280M products was the BsmBI endonuclease.

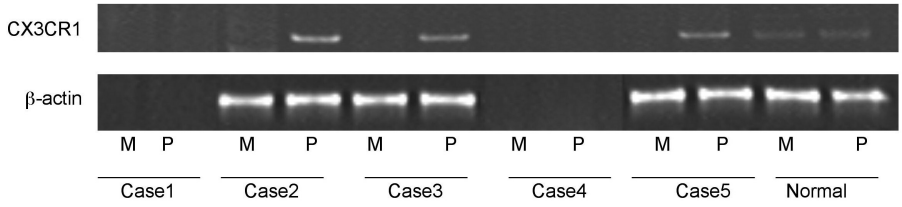

Fig. 2. Autoradiography showing *CX3CR1* mRNA expression in perimacular but not macular cells of three AMD cases with *CX3CR1* M280. *CX3CR1* transcripts were detected in both macular (M) and perimacular (P) cells of a normal eye. Two other cases (Thr/Thr and Thr/Met) did not yield RT-PCR products.

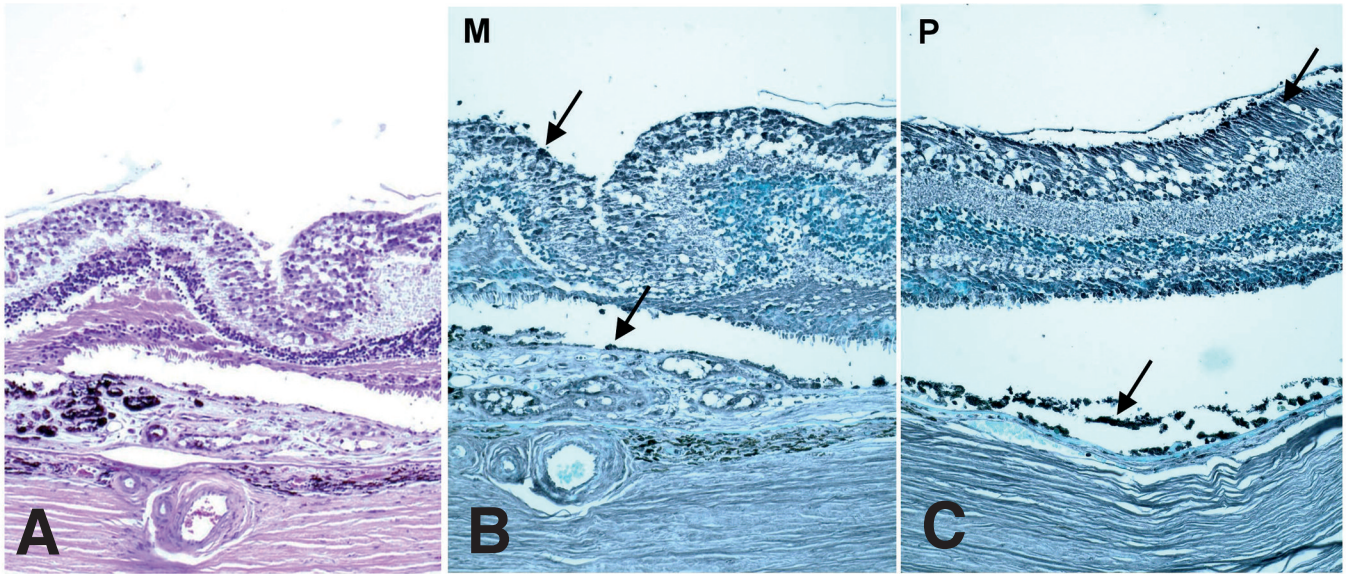

Fig. 3. Photomicrograph showing expression of CX3CR1 on Muller cells, photoreceptors and RPE cells. AMD is confirmed in the hematoxylin and eosin slide **(A)**. Lower expression is found in the macula **(B)** as compared to the perimacula **(C)** of the same eye with AMD (B and C, avidin-biotin-complex immunoperoxidase, original magnification, x 100).

these patients. Further demographic information was unavailable for 5 of these 32 cases. General demographic information of the 27 known patients includes a gender distribution of 20 females and 7 males and a race distribution of 26 Caucasians and 1 African American. Histopathology diagnosed 18 of these cases with neovascular AMD and 14 without neovascular AMD. Sixteen of the 32 cases were found to be *CX3CR1* M280 carriers: 13 cases were heterozygous (Thr/Met; 8 with neovascular AMD and 5 without neovascular AMD) and 3 were homozygous for the variant allele (Met/Met; two with neovascular and one without neovascular AMD). The M280 allele frequency was 29.7%. Out of the 16 M280 carriers, 10 had neovascular AMD (allele frequency of 33%) and 6 had non-neovascular AMD (allele frequency of 25%). Although there was a slightly higher prevalence of the M280 allele in the neovascular AMD cases, our findings suggest that this SNP may not correlate well with phenotype. In other words, *CX3CR1* M280 carriers could belong to either the neovascular or non-neovascular AMD group.

Lowered expression of CX3CR1 in the AMD macula

Four (3 neovascular and 1 without neovascular AMD) of the 5 NEI cases carried the *CX3CR1* M280 (Thr/Met) allele. The remaining case (with neovascular AMD) was Thr/Thr in genotype (Fig. 1). *CX3CR1* messengers were successfully recovered from 3 (2 with neovascular and 1 without neovascular AMD) of these 5 cases. Of these three cases, each carried the M280 allele (Thr/Met) and demonstrated an absence of *CX3CR1* mRNA expression in the macula as compared to the peripheral retina. In contrast, *CX3CR1* mRNA expression in the pairs of normal eyes (with ages of 70 and 78 years) detected no difference of *CX3CR1* transcripts between the macular and perimacular and peripheral retina regions (Fig. 2).

CX3CR1 stained positively at the RPE, Müller cell, microglia and outer segment of the photoreceptors in the retina of normal eye. However, positive reactivity of CX3CR1 was decreased in number and intensity in the macula of the eyes affected by both with and without neovascular AMD (Fig. 3). No changes in intensity, number, or staining pattern were found in the peripheral retina in the eyes with AMD as compared to the normal eyes.

Discussion

In this study, we have successfully applied a combination of microdissection and PCR-RFLP techniques in order to detect *CX3CR1* SNPs on archived, paraffin embedded slides. In addition, we have also successfully detected CX3CR1 transcripts and protein expression in multiple cell types within the retina using microdissection combined with RT-PCR and immunohistochemistry. Due to limitations imposed by the small amount of DNA or RNA yielded via microdissection from the archived slides, we were unable to perform real time quantitative PCR. However, we were able to compare semi-quantitative PCR products to ß-actin gene expression. This combination of techniques allows us to more thoroughly analyze the molecular pathology of AMD as well as better understand disease pathophysiology.

This study demonstrated a possible association between the *CX3CR1* polymorphism (T280M) and AMD through analysis of archived eye sections. A higher allele frequency of the M280 allele was found in our AMD cases (29.7%) as compared to frequency found in the controls (11%) and general population (10-18%) (Liu et al., 2003; McDermott et al., 2003) reported by LocusLink (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=1524). This association was also observed in our recent AMD study, in which we reported an M280 allele frequency of 11.0% (allele, n=210) in the control population and 19.4% (n=170) in the AMD case group (Tuo et al., 2004b). In the previous study, we also observed consistent variation in expression levels of the transcripts and protein in matched normal subjects with different genotypes (Tuo et al., 2004b). In the present study, we have further shown a lowered expression of CX3CR1 messenger and protein in the macular area as compared to the peripheral and perimacular retina of the eyes with AMD. These findings support that CX3CR1, a chemokine receptor, may play a role in the development of AMD.

Chemokines are low molecular weight (8-10 kDa) peptides or glycopeptides that mediate leukocyte chemotaxis and increase cellular adhesion (Taub and Oppenheim, 1994; Homey et al., 2002). These essential properties are required in order to efficiently direct cells to sites of infection or inflammation. Chemokine molecules are classified into four distinct families based on the positioning of the first two of four conserved cysteine residues (Zlotnik and Yoshie, 2000). These 4 families are classified as CXC, CC, XC, and CX3C. The receptors for these molecules are seven-transmembrane domain G protein-coupled receptors (Zlotnik et al., 1999). Chemokines are involved in not only inflammatory and immune responses, but in other disease processes as well such as malignancy, angiogenesis, and hematopoiesis.

To date, fractalkine/CX3CL1, also known as neurotactin, is the only identified CX3C chemokine. CX3CL1 is widely expressed in various organs such as the eye (Foxman et al., 2002) brain, lung, heart, kidney and small intestine (Cotter et al., 2002). CX3CR1, the only receptor of CX3CL1, is expressed on different leukocytes such as macrophages as well as on microglia and astrocytes (Mizuno et al., 2003). In this study, we have demonstrated intensive staining of CX3CR1 on the RPE, Müller cells, glial cells, and photoreceptors in the retina of the normal eye.

Strong up-regulation of *CX3CR1* in macrophages has been reported in response to experimental ischemic-reperfusion brain injury and glormerulonephritis (Feng

et al., 1999; Tarozzo et al., 2002) as well as in the affected skin of patients with atopic dermatitis (Echigo et al., 2004). These findings suggest that CX3CL1 plays a central role in the trafficking of macrophages into tissues with lesions that release CX3CL1. In this context, expression of CX3CR1 on macrophages is important for their recruitment to the appropriate location.

The majority of DNA sequence variation in the human genome is in the form of SNPs (Risch and Merikangas, 1996; Wang et al., 1998; O'Brien et al., 1999). SNPs are defined as persistent substitutions of a single base with a frequency of more than 1%. The screening of common SNPs has become an attractive tool in the exploration of the genetic component of complex diseases such as AMD (Risch, 2000; Wright, 2001; Amouyel, 2002; Hyman and Neborsky, 2002; Stone et al., 2004; Zareparsi et al., 2004).

Recently, a number of SNPs in coding areas have been identified in *CX3CR1*. These SNPs have been shown to alter susceptibility to both HIV infection and arteriosclerosis (Faure et al., 2000; McDermott et al., 2001; Moatti et al., 2001). The two SNPs that have been implicated include a valine to isoleucine substitution at position 249 (V249I) and a threonine to methionine substitution at position 280 (T280M). These two polymorphisms are in complete linkage disequilibrium, meaning that chromosomes possessing the M280 polymorphism also have the I249 variation. The M280 polymorphism is associated with a decreased binding affinity for the CX3CL1 ligand (Faure et al., 2000). The V249I SNP has been correlated with a 35% decrease in cell surface receptor number (Moatti et al., 2001). In addition, two *CX3CR1* promoter SNPs have been found in weak linkage disequilibrium with the T280M and V249I SNPs. Another promoter SNP has also been shown to be in moderately strong linkage disequilibrium with both coding SNPs (DeVries et al., 2003).

In this study, we have found an increase in *CX3CR1* M280 allele frequency (29.7%) in the 32 pathologically diagnosed AMD cases as compared to the general control population (10-12%). We have also found a decrease in both CX3CR1 protein and transcript expression in the macula as compared to the peripheral retina of the AMD eyes. There was no significant difference in distribution between the two AMD phenotypes with and without neovascularization. In contrast, CX3CR1 transcription and protein are same in both macular and peripheral retina in normal controls. While discussion of the pathogenic mechanisms associated with the *CX3CR1* polymorphisms and AMD development may be still speculative at the present time, the potential involvement of macrophages in AMD appears to be a logical focal point for future studies.

Macrophages have been well recognized as active participants in neovascular AMD development (Kimura et al., 1999; Grossniklaus et al., 2000; Espinosa-Heidmann et al., 2003; Tsutsumi et al., 2003). Macrophages have also been shown to phagocytize pigment and debris and either migrate into the retina or form clumps in the photoreceptor region (Killingsworth et al., 1990). Therefore, macrophages may also accumulate as a result of areolar atrophy without neovascularization.

On the other hand, it has been demonstrated that an increased amount of deposit in Bruch's membrane is associated with the presence and severity of AMD (van der Schaft et al., 1992; Green, 1999; Spraul et al., 1999). Macrophages, like other cell types such as endothelial cells, aortic smooth muscle cells, neuronal cells, and keratinocytes (Zingg et al., 2000), possess scavenging receptors. Previous studies on animals that completely lack CCL2, a more ubiquitous chemoattractant for macrophages, or its receptor, CCR2, demonstrated a marked reduction in the number of macrophages in the tissue thereby contributing to the development of both drusen and CNV (Ambati et al., 2003b). In other words, in the healthy eye, normal choroidal macrophage activity may contribute to drusen disposal thereby actually preventing AMD formation (Ambati et al., 2003a; Forrester, 2003).

Whether or not CX3CL1, in its function as an adhesion molecule and chemoattractant for macrophages that bear its receptor, CX3CR1, is involved in directing macrophages into Bruch's membrane is still unknown. However, we hypothesize that aberrant CX3CR1 function may result in inadequate removal of these deposits thereby contributing to the development of AMD (Tuo et al., 2004b). The current pathological study supports this hypothesis. Further studies investigating the functional significance of *CX3CR1* in AMD development are currently underway in our laboratory.

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