A CX_3CR1 Genotype Associated with Retinal Vasculitis in Patients in the United Kingdom

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Purpose. To investigate whether polymorphisms in the gene encoding the chemokine receptor CX_3CR1, which has been linked to changes in functional ligand-binding activity, are associated with retinal vasculitis (RV) in a cohort of patients in the United Kingdom.

Methods. DNA was prepared from whole blood of 126 patients with RV and 95 healthy individuals by a standard salting-out procedure. Two polymorphisms, V249I and T280M, were analyzed by multiplex polymerase chain reaction–sequence-specific primers (PCR-SSPs).

Results. There was no significant difference between the prevalence of V249 or I249 variants in patients with RV or in control subjects. By contrast, the 280M variant was significantly raised in patients compared with control subjects ($P = 0.01$), the IV/MT haplotype was also more prevalent in patients with RV than in control subjects ($P = 0.006$), and the I249/M280 haplotype was associated with retinal vasculitis ($P = 0.01$). The 280M variant was significantly associated with the nonischemic form of RV compared with healthy control subjects ($P = 0.009$).

Conclusions. Polymorphisms related to a functional decrease in ligand binding activity of CX_3CR1 are associated with disease in U.K. patients with retinal vasculitis. CX_3CR1 and its ligand CX_3CL1 have been implicated in leukocyte adhesion and neuronal protection. Changes in the activity of this interaction may have a role in the pathogenesis of RV. (*Invest Ophthalmol Vis Sci.* 2006;47:2966–2970) DOI:10.1167/iovs.05-1631

Uveitis is a generic term used to define a group of potentially sight-threatening disorders that usually affect young adults and is classified according to the anatomic area of the eye that is affected. Uveitis can be caused by isolated inflammatory disease, such as retinal vasculitis (RV) affecting the retinal vessels or can be part of a more widespread inflammatory disorder in which the eye is involved with other organs (Behçet's disease, sarcoidosis). It is generally considered to be an autoimmune, cell-mediated, organ specific disease based on the findings of autoreactive T cells and antibodies in patients, the response of the disease to immunosuppression, and the existence of experimental models that share many of the clinical and immunologic features of the human disease.[1]

Uveitis is characterized by leukocyte infiltration of retinal tissue.[2] For migrating leukocytes to enter ocular, tissue they must cross the complex blood–retinal barrier (BRB) formed by retinal endothelial and pigment epithelial cells. A critical question in the pathogenesis of uveitis is how leukocytes cross the normally impervious BRB and thus initiate subsequent pathologic damage.

Chemokines are a group of small (8–10 kDa), secreted proteins that were initially identified by their ability to attract leukocytes. These molecules induce leukocytes to migrate along concentration gradients, and modulate interactions with endothelial cells through the upregulation and reversible activation of integrins.[3] One such chemokine, (or fractalkine) CX_3CL1, has a structure that differs from that of other chemokines, as it is bound directly to cell membranes via a mucin stalk.[4] CX_3CL1 is widely expressed in the rodent brain and located principally in neurons, and the expression of its receptor, CX_3CR1, has been shown on microglia and neurons.[5] CX_3CL1 expression on endothelium has been described; and, under flow conditions, CX_3CL1 captures leukocytes. Cell lines transfected with other chemokines attached to the CX_3CR1 mucin stalk show increased binding due to a slower release rate from the linked receptor than from the natural form.[6]

CX_3CL1 has been found in the eye. Cadaveric iris and retinal explants constitutively express CX_3CL1 protein in microvascular endothelial and several stromal cell types. Similarly both expressed CX_3CL1 mRNA. TNF upregulated CX_3CL1 mRNA in iris explants and in cultured iris and retinal endothelial cells. TNF and IFNγ increase CX_3CL1 binding to iris endothelial cells (ECs) both separately and synergistically, whereas IL-4 significantly decreases binding, The cytokine results suggest that a Th1 response would upregulate CX_3CL1 expression whereas a Th2 response would downregulate it.[7] It has been suggested that CX_3CL1 may be involved in the mechanisms of immune surveillance of ocular tissue.

Recent studies have identified two single nucleotide polymorphisms (SNPs) in the gene encoding CX_3CR1; 839 C→T (rs3732378; T280M) and 745 G→A (rs3732379; V249I). Patients homozygous for the variant haplotype, I249-M280, progress to AIDS more rapidly than patients with other haplotypes, possibly due to compromise of normal immune responses, which accelerates progression to disease.[8,9] By comparison, a reduced prevalence and severity of coronary artery disease (CAD) was associated with I249-M280, compared with individuals homozygous for V249, and it has been postulated that the effect was mediated through a reduced recruitment of inflammatory cells to the atherogenic site.[10,11]

Against this background, we hypothesized that the form, severity, and outcome of disease might differ in patients with retinal vasculitis with the I249-M280 haplotype compared with other variants. We specifically looked for associations between the haplotypes and the form of disease (whether or not retinal

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capillary closure was present) and for associations with disease outcome at five years after presentation. The results show that the M280 was significantly raised in patients with nonischemic RV and that the I249-M280 genotype was associated with disease.

Materials and Methods

The study population ($n = 126$) was taken from patients attending the Medical Eye Unit, St. Thomas' Hospital, London. Blood samples were collected by venipuncture. All patients had been followed up for at least 5 years. These patients were classified as having idiopathic retinal vasculitis on the basis of ophthalmic examination and fluorescein angiography. To be included, patients had to show evidence of intraocular inflammation with macroscopic (or fluorescein angiographic) involvement of retinal veins. Accordingly, the study population included both patients with intermediate and posterior uveitis. Patients with clinically definite multiple sclerosis (MS), sarcoidosis, Behçet's disease, seronegative arthropathy, inflammatory bowel disease, or infectious or neoplastic uveitis at baseline, or who developed these diseases during follow-up were excluded on the basis of clinical history, systemic examination and relevant investigations. Patients with local ocular inflammatory syndromes such as Fuchs' heterochromic cyclitis and any form of choroiditis were also excluded, as were patients who had never shown evidence of intraocular inflammation, A good outcome was determined as visual acuity (VA) better than 20/40 in both eyes at 5 years after presentation. In all cases with a bad outcome (VA <20/40 in both eyes), there was objective evidence of irreversible macular dysfunction from fluorescein angiography (e.g., persistent cystoid macular edema, unresponsive to intravitreal steroids; or macular ischemia; or epiretinal membranes). All outcome analysis was performed before CX_3CR1 genotyping. For comparison, in the genotyping studies, local healthy control subjects ($n = 95$) were used. Control subjects were age, sex, and ethnically matched but had not undergone formal ophthalmologic evaluation. This study was approved by the St. Thomas' Research Ethics Committee, and all patients gave informed consent in accordance with the Declaration of Helsinki.

Cytokine Gene Polymorphisms

DNA was prepared from venous blood obtained from patients and control subjects by proteinase K digestion and high salt extraction and stored at −70°C until use.[12] polymerase chain reaction–sequence-specific primers (PCR-SSPs) were designed to amplify between the nonsynonymous single nucleotide polymorphisms C839T (T280M) and G745A (V249I) of the CX_3CR1 gene giving amplification products of 135 base pairs: forward primer 1, 5′-CTTCTggACACCCTAACAAg-3′; forward primer 2, 5′-TCTTCTggACACCCTACAACA-3′; reverse primer 3, 5′ AACAATggCTAAATgCAACCg-3′; and reverse primer 4, 5′ AACAATggCTAAATGCAACCA 3′.

By using the four reactions between primer 1 and primers 3 and 4, and primer 2 with primers 3 and 4, the haplotypes were unequivocally derived. The conditions for amplification were 96°C, 25 seconds; 70°C, 45 seconds; and 72°C, 30 seconds; for five cycles, 96°C, 25 seconds; 65°C, 45 seconds; and 72°C, 30 seconds for 20 cycles, and finally 96°C, 25 seconds; 55°C, 60 seconds; and 72°C, 120 seconds for five cycles. Associations with disease, disease type and outcome were calculated by both allelic frequency and haplotype analysis.

Analysis of Data

Associations between CX_3CR1 variants, ocular disease, disease type, and outcome, were identified by χ^2 analysis, using Yates correction with the odds ratio and 95% confidence intervals calculated.

Results

The CX_3CR1 has two SNPs in the coding region that were analyzed for association with disease in patients with retinal vasculitis. The 249 and 280 genotype was in Hardy-Weinberg equilibrium in the patient and control groups, when considered separately, and the haplotype frequency was similar to that reported in other studies.[8,9] There was no significant difference in the frequencies of V249 and I249 between patients and control subjects. By comparison, the M280 variant was significantly associated with patients with RV compared with healthy control subjects (49/252 [19%] vs. 20/190 [11%]; $\chi^2 = 5.9$, $P = 0.01$; OR, 2.05). This increase was due to the higher number of heterozygotes in the RV patient population (35/126 [28%] vs. 12/95 [13%]) and the concordant fewer wild-type homozygotes (84/126 [67%] vs. 79/95 [83%]; Table 1).

Further analysis showed an increase in haplotype 6 (IV/TM) in patients with RV compared with all other haplotypes (33/126 [25%] vs. 10/95 [11%]; $\chi^2 = 7.5$, $P = 0.006$; OR, 3), and a consequent decrease in haplotype 1 (VV/TT; 71/126 [56%] vs. 63/95 [66%]; Table 2).

Linkage disequilibrium has shown that only three amino acid combinations are formed by CX_3CR1 gene polymorphisms (V249/T280, I249/M280, I249/T280) as M280 is rarely found in the absence of I249 (none in this study), although the opposite does not hold true. Analysis of the three haplotypes showed an increase in the I249/M280, (49/252 [19%] vs. 19/190 [10%]; $\chi^2 = 6.7$, $P = 0.01$; OR, 2.2) in patients compared with control subjects (Table 3).

Clinically, RV is classified as ischemic or nonischemic on the basis of the presence of retinal capillary closure revealed by fluorescein angiography. When analyzed by form of disease, male patients were significantly more likely to have ischemic disease, although females represented the largest gender in the study ($\chi^2 = 9.6$, $P = 0.002$, OR, 5.2; Table 4). Furthermore, analysis of each group showed that patients with ischemic disease were never homozygous for the mutant alleles, and the I249 variant was underrepresented in the ischemic population and overrepresented in the nonischemic patients, although these results did not reach significance (ischemic versus nonischemic patients; $\chi^2 = 0.8$, $P = 0.4$). By comparison, the M280 variant was significantly raised in patients with nonischemic RV compared with the control ($\chi^2 = 6.7$, $P = 0.009$, OR, 2.2) (Table 5). Therefore, nonischemic patients with RV were more likely to possess the M280 variant and be female; however, when analyzed on the basis of gender there was no significant difference in the distribution of the either variant in males and females (data not shown). Therefore M280 was directly linked to the nonischemic form of RV.

Table 1. Haplotype Analysis of CX_3CR1 Polymorphisms V249I and T280M

Haplotype	Control ($n = 95$)	RV Patients ($n = 126$)
V249I		
VV	63 (66)	71 (56)
VI	25 (26)	45 (36)
II	7 (8)	10 (8)
V	151 (79)	187 (74)
I	39 (21)	65 (26)
T280M		
TT	79 (83)	84 (67)
TM	12 (13)	35 (28)
MM	4 (4)	7 (5)
T	170 (89)	203 (81)
M	20 (11)	49 (19)

The 280M variant is associated with RV ($\chi^2 = 5.9$ $P = 0.01$; OR 2.05; 95% CI 1.14–3.7). Data are number (percentage).

Table 2. Haplotype Analysis of CX_3CR1 I249V and M280T in Patients with RV

Haplotype* 249/280	Control Subjects (n = 95)	RV Patients (n = 126)
(1) VV/TT	63 (66)	71 (56)
(2) II/TT	2 (2)	1 (1)
(3) II/MM	4 (4)	7 (6)
(4) II/TM	1 (1)	2 (2)
(5) VI/TT	15 (16)	12 (10)
(6) IV/TM	10 (11)	33 (25)*

Data are the number (percentage).

* Haplotype 6 is significantly associated with disease (χ^2 = 7.5; P = 0.006 OR 3; 95% CI 1.3–7).

Finally, analysis on the basis of the 5-year follow-up showed no significant difference with either variant and good or bad clinical outcome (data not shown).

Discussion

Retinal vasculitis is characterized by leukocyte infiltration of normally impervious retinal tissue leading to edema, death of photoreceptor cells and decreased visual acuity. The molecules involved in breakdown of the blood–retinal barrier include CD54, CCL2, CCL5, and CCL4.[13,14] Our data show that SNPs in the gene encoding CX_3CR1 are associated with retinal vasculitis. Expression of the M280 variant and a haplotype encoding a complex variant form were significantly more prevalent in patients with RV than in control subjects. Of the three possible haplotypes formed by these SNPs, the mutant haplotype, was associated with disease. The M280 variant was found to be associated with nonischemic RV, but did not associate with gender or outcome in these patients. As M280 was always found with I249, but the reverse was not true, it is not possible to state definitively which allele is most important.

We have used the term retinal vasculitis to define our population. It is our belief that the definition that we have given, which includes patients with intermediate and posterior uveitis without choroidal or retinal pigment epithelial involvement, is reasonable in the absence of criteria that define a separate nosologic entity.[15] We specifically excluded any patient with choroiditis, because the natural history and systemic associations of patients in this category are uncertain. There are potential sources of bias in this study that relate to the patient population. First, there may have been referral and selection bias. Our patients were derived from a tertiary referral population, and although they were observed as a routine for extended periods, those who had mild disease or in whom disease had regressed may have been lost to follow-up. Accordingly, only patients with more severe disease may have been included in the analysis. Second, because this was a review of case records, it is possible that further improvements in visual acuity after 5 years may have been achieved with more aggressive treatment (i.e., the best recorded visual acuity at the end of 5 years of follow-up was not necessarily the best possible). However, in all cases, the patients were seen during the fifth year by senior ophthalmologists experienced in the management of intraocular inflammation, and our policy of administering intraocular steroids when conventional treatment had not worked make this less likely. Third, although unlikely, it is still possible that patients will develop an associated systemic disease, even at this stage (i.e., the reported population may not be truly idiopathic). Multiple sclerosis has been reported to occur after 7 years of follow-up, and a recent study showed that the mean time from the onset of uveitis to development of MS was 8.5 years.[16,17]

Table 3. The Three Complex Haplotypes Found in the Study

Haplotype	Control Subjects	RV Patients
V249/T280	151 (80)	187 (75)
I249/T280	20 (10)	16 (6)
I249/M280	19 (10)	49 (19)*

The I249/M280 haplotype was significantly increased in the RV patients group (χ^2 6.7 P = 0.01; OR 2.2; 95% 1.2–4).

Table 4. Clinical Form of RV Based on Retinal Fluorescein Angiography

	Male	Female
Ischemic	15	6
Nonischemic	34	71

The results are the number of patients with each form of RV separated for gender. The ischemic form of RV was more prevalent in male patients (χ^2 = 9.6; P = 0.002, OR 5.2; 95% CI 1.7–16.7).

The effect of polymorphism in CX_3CR1 in relation to function is complex. Initial studies with soluble CX_3CL1 showed that I249-M280$^+$ cells display impaired chemokine binding, calcium response, and chemotaxis, due to a reduction in the total number of binding sites that reduced CX_3CL1 binding affinity.[8,10,18] Recent studies have not confirmed these findings and have demonstrated increased binding of CX_3CR1 I249-M280$^+$ cells to membrane-bound CX_3CL1.[19,20] The discrepancies in these results may be due to experimental differences, or may define a loss of function of CX_3CR1 in the chemotactic response to soluble CX_3CL1, and gain of function to the membrane-bound form. These mechanisms require further study.

The potential role CX_3CR1 in retinal disease is equally complex. $CX_3CL1^{-/-}$ mice did not show any overt behavioral abnormalities or gross changes in the brain and had a normal hematologic profile except for a decrease in the number of F4/80 cells. Despite this decrease there was no difference in response to thioglycollate, suggesting there was no inherent loss of migratory function in the macrophage population. Similarly, delayed-type hypersensitivity (DTH) responses to keyhole-limpet hemocyanin (KLH) were the same. Finally, no difference in responses to colitis induction or infection with *Listeria monocytogenes* was seen.[21] Therefore, a direct role for CX_3CR1 in leukocyte trafficking into the retina during inflammation cannot be confirmed.

Recent studies have identified two subsets of blood monocytes based on their expression of CX_3CR1. High expression

Table 5. CX_3CR1 T280M Genotype and Form of Retinal Vasculitis with Patients Separated into Ischemic and Nonischemic Groups Based on Retinal Angiography

CX_3CR1 T280M	Ischaemic (n = 21)	Nonischaemic (n = 105)	Control (n = 95)
MM	—	7	4
MT	6	29	12
TT	15	69	79
M	6	43	20
T	36	167	170

The results are the number of patients with each form of RV and controls. 280M expression is significantly raised in nonischemic patients versus control (χ^2 6.7; P = 0.009; OR 2.2; 95% CI 1.2–4).

identified a population associated with immune surveillance and precursors of resident tissue macrophages. CX_3R1^{lo} have been found to infiltrate sites of inflammation, possibly via CCR2, and may be precursors of circulating dendritic cells. Moreover, binding of CX_3L1 was suggested to provide a survival signal to CX_3R1^{hi} monocytes.[22] In support of this CX_3CR1^{+} microglia express CD95 and CD95L, and CX_3CL1 treatment of microglia maintains cell survival and inhibits Fas-ligand induced apoptosis in vitro. Survival is related to inhibition of the proapoptotic molecules BAD and BID and upregulation of Bcl-x_L.[23] Therefore, it is possible that CX_3CR1 M280, which is related to reduced function, affects recruitment of CX_3R1^{hi} resident monocyte precursors, but has little influence on inflammatory monocytes. Of note, in support of this hypothesis, monocytes have a sentinel protective role against age-related macular degeneration.[24] An association between CX_3CR1 I249 and M280 has been described in patients with AMD compared with control subjects. Moreover, retinal cells microdissected from AMD and normal archival tissue were analyzed in a recent study. The M280 allele was found at a significantly higher frequency in cells from patients with AMD than in the normal population. Moreover, cells from patients with AMD had less CX_3CR1 transcript and protein, which suggests that both altered function and expression of CX_3CR1 contribute to AMD, possibly through a decrease in chemoattraction of inflammatory cells.[25,26]

A final possibility is that CX_3CR1 is involved in neuronal cell protection. It is strongly expressed on Müller cells in human retina.[7] Both CX_3CR1 and CX_3CL1 are highly expressed in human and rodent brain.[5] Resident microglia in the parenchyma, the choroid plexus, and meninges express CX_3CR1, neurons express both receptor and ligand, whereas astrocytes and oligodendrocytes express neither. In a prion-induced model of brain inflammation, upregulation of CX_3CL1 and CX_3CR1 expression in astrocytes and microglia, respectively, correlated with neuronal loss. In contrast, acute activation of resident microglia and astrocytes resulting from intracerebral LPS injection did not result in a significant increase in either CX_3CL1 or CX_3CR1, and intracerebral injection of CX_3CL1 induced microglia activation without the recruitment of blood-borne cells.[27] Moreover, CX_3CR1-CX_3CL1 interaction inhibited the release of TNF, IL-6, and nitric oxide by monocytes and activated microglia, and reduced neuronal cytotoxicity by neurotoxins.[28,29] Therefore, it is possible that the differential function of these molecules in acute and chronic neurodegeneration reflects the local cytokine environment, which switches microglia from a pro- to anti-inflammatory phenotype and protects against neuronal cell death. The effect of CX_3CR1 polymorphisms on retinal microglia have yet to be elucidated.

Our data that demonstrate an association between a haplotype linked to reduced function for CX_3CR1 are intriguing because of the potentially opposing nature of CX_3CR1-mediated responses. If CX_3CR1 were involved directly in leukocyte trafficking into retinal tissue, then a reduced function would be beneficial to patients with RV. However, if mechanisms such as those involved in the protection of neuronal cells (i.e., photoreceptor cells) from cell death are involved in retinal vasculitis, expression of a genetic haplotype of CX_3CR1 associated with reduced function would lead to more destructive disease and loss of visual acuity. Further functional work is needed to assess these two possibilities.

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