Internal Carotid Artery Occlusive Disease and Polymorphisms of Fractalkine Receptor CX3CR1
A Genetic Risk Factor

Giorgio Ghilardi, MD; Maria Luisa Biondi, MD; Olivia Turri, PhD; Emma Guagnellini, MD; Roberto Scorza, MD

Background and Purpose—Fractalkine (FKN), a chemokine expressed by inflamed endothelium, induces leukocyte adhesion and migration via the receptor CX3CR1. The polymorphisms V249I and T280M affect receptor expression and function. The role of FKN in atherosclerosis has been recently demonstrated. The aim of this study was to investigate a possible association between CX3CR1 polymorphisms and increased risk of internal carotid artery (ICA) occlusive disease.

Methods—We studied 108 patients consecutively recruited for ICA occlusive disease, 84 of whom underwent operation for carotid endarterectomy, and 204 subjects without ICA occlusive disease (controls). Polymorphic genotypes were determined by polymerase chain reaction and sequencing analysis.

Results—The adjusted odds ratio (OR) associated with the presence of the M280 (TM/H11001 MM versus TT genotype) was 0.55 (95% CI: 0.29 to 0.99; \( P = 0.037 \)). Therefore, this allele is associated with a reduced risk of ICA occlusive disease. No significant differences were observed in I249 distribution. The frequency of I249 allele was significantly higher in cases of hard plaques, which are considered more stable than soft ones (OR: 0.38; 95% CI: 0.13 to 1.05; \( P = 0.037 \)). Multiple logistic regression analysis using the common risk factors and the I249 and M280 allele variants revealed that the M280 allele was an independent risk factor for ICA stenosis (\( P = 0.047 \)).

Conclusion—The results show that the CX3CR1 M280 is an independent genetic risk factor for ICA occlusive disease and that I249 is involved in the stability of carotid plaques. Even if obtained from a relatively limited patient series, these results might have relevant implications for treatment of ICA stenosis and possibly prevention of carotid related stroke. Further prospective cross-sectional studies are needed to confirm these results. (Stroke. 2004;35:1276-1279.)

Key Words: carotid stenosis • carotid endarterectomy • chemokines, CX3C • receptors, chemokine polymorphisms, single nucleotide

Atherosclerosis is a multifactorial process that involves inflammation in response to progressive vascular injury. A cardinal feature is inflammation of the vessel wall arising from interactions of leukocytes with vascular endothelial cells, smooth muscle cells, and fibroblasts. At the molecular level, interactions among these cell types are regulated by cytokines, adhesion molecules, and chemoattractants. Chemokines are a large family of chemoattractants that direct migration of leukocytes from the blood to sites of inflammation. Fractalkine (FKN) and its receptor, CX3CR1, have recently emerged as particularly interesting candidates for susceptibility to atherosclerosis as shown by expression, functional, and epidemiological data. FKN is a unique chemokine because it exists in soluble form and membrane-anchored form. Membrane-bound FKN directly mediates the capture and firm adhesion of CX3CR1-expressing leukocytes, thus providing a novel pathway for leukocyte activation. Soluble FKN has leukocyte chemotactic activity. Faure et al have previously reported 2 common polymorphisms of CX3CR1 that are in complete linkage disequilibrium (V249I and T280M). They were associated with reduced prevalence of acute coronary endothelial dysfunction in an National Institutes of Health (NIH) cardiac catheterization cohort and with reduced prevalence of acute coronary events in the Accidents Coronaires Aigus Bichet (ACABI) cohort from France. Noticeably, functional CX3CR1 analysis showed that FKN binding was reduced in peripheral blood mononuclear cells (PBMCs) from patients with human immunodeficiency virus (HIV) homozygous for the I249M280 haplotype. Internal carotid artery (ICA) occlusive disease has been recognized as a major cause of stroke. Carotid endarterecto-
my has been validated by several large trials as effective in prevention of stroke secondary to severe ICA stenosis.13–16

Rupture of atherosclerotic plaques is the predominant underlying process in the pathogenesis of acute coronary syndromes and strokes.17,18 Interactions, within the ICA stenosing plaque, between connective tissue and the cells embedded into the fibrous cap overlaying the inner core, which is filled with lipids and necrotic debris, appear to determine the history of the ICA stenosis, particularly complications that are recognized cause of stroke, such as plaque rupture or ulceration, intraplaque hemorrhage, and luminal thrombosis.17,18 Although the morphology of ruptured plaques is well-described, specific markers to identify in vivo ruptured plaques underwent operation for carotid endarterectomy. Stability in human subjects candidates for or who have undergone operation for carotid endarterectomy.

Subjects and Methods
We genotyped 108 subjects affected with ICA occlusive disease consecutively referred to our vascular surgery unit and 204 unsolicited volunteer outpatients (controls) consecutively referred to our vascular ultrasound laboratory, with no evidence of ICA occlusive disease at ultrasound color Doppler (USCD) examination. Informed consent was obtained from patients and controls. All 312 participating subjects underwent basic vascular evaluation, including clinical vascular examination, thorough USCD of the accessible arterial tree, and an electrocardiogram at rest. Patients underwent additional neurological evaluation and cerebral computed tomography (CT) to assess symptoms or cerebral infarction related to ICA stenosis. Among patients, 35% had history of coronary artery disease, 2% had clinical evidence of peripheral arterial occlusive disease, and 2% had incidental diagnosis of abdominal aortic aneurysm. Among controls, the percentages were 32%, 2.5%, and 1%, respectively.

Hypertension was defined according to the Canadian Medical Association guidelines for the management of hypertension.19 Smoker definition included ex-smokers and active smokers. Hypercholesterolemia was defined as elevated total serum cholesterol levels >200 mg/ dL.

The degree of carotid stenosis was assessed by USCD (Philips ATL HDI 5000 SonocT system; 7.5 to 10 MHz linear transducer) and confirmed by multidetector helical angio-CT (MHACT) (General Electric LightSpeed QXi; G.E. Advantage Windows for image postprocessing). In this article, the degree of stenosis is reported in percentages equivalent to NASCET definition.20,21 According to the American Heart Association Scientific Statement on carotid endarterectomy,22 84 patients with >50% symptomatic stenosis and >70% asymptomatic stenosis were assigned to surgery and 24 of the asymptomatic subjects did not undergo operation. Carotid endarterectomies were performed by standard eversion technique. Plaque specimens (n=84) were inspected in the operative room by an experienced pathologist and the macroscopic observations were recorded within the operation report file. Carotid plaques were classified according to the American Heart Association statement on classification of advanced atherosclerotic lesions.23 For the purpose of this article, plaques were defined as soft or hard, according to the relative content of lipids, debris or intraplaque hemorrhage, and calcium. The macroscopic examination was then matched to the preoperative MHACT examination and a substantial agreement was found in any instance. Given the ability of MHACT to accurately estimate the calcium content of the plaque,24 a cutoff of 33% calcium content was established to define soft plaques (<33% calcium content) and hard plaques (>33% calcium content). Patients with bilateral lesions were considered only once in this study (first operation).

Whole blood (3 mL) from patients and controls was collected into potassium EDTA. DNA was prepared with Istagene Matrix extraction kit (Bio-Rad Laboratories). The polymerase chain reaction for CX3CR1 was performed in a total volume of 25 μL with 5 μL of extracted genomic DNA, 100 μmol/L of dATP, dGTP, dTTP, and dCTP, 1.5 mmol/L of MgCl₂, 1 U of Taq polymerase, the 2 primers, forward and reverse, each at a concentration of 80 nm. The primers were designed with Primer Express software. Forward primer is 5′-AGAATCCATCCAGGCTGGTTTCC-3′ and reverse primer is 5′-CACAGGACGCCAGGATT CCC-3′. The polymerase chain reaction starts with 10-minute incubation at 95°C to activate the enzyme, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The amplification was verified on an agarose gel (2%) followed directly by sequencing with an automatic sequencer in fluorescent DNA capillary electrophoresis (ABI Prism 310; Applied Biosystems).

Differences between groups were examined by χ² test. Odds ratios (ORs) (approximate relative risk) were calculated as an index of the association of the V249I and T280M genotype with each phenotype. For each OR, 2-tailed P and 95% CIs were calculated. Multiple logistic regression analysis was used to calculate the OR of ICA stenosis and its 95% CI in subjects exposed to specific risk factors. Only the factors that were significantly associated with the development of carotid plaque on univariate analysis were included in the logistic regression analysis. All statistical analyses were 2-sided and were performed with Stata Statistical Software (Stata Corporation). Statistical analysis was assumed significant for P<0.05.

Results
Allele frequencies in control and patient populations were in Hardy–Weinberg equilibrium for both CX3CR1 polymorphisms. Although gender frequency was similar between the 2 groups (P=0.71), controls were slightly younger (mean ages: patients 68±6 years; controls 65±7; P=0.02) (Table 1).

The adjusted ORs associated with the presence of the M280 (TM+MM versus TT genotype) and I249 alleles (V1+II versus VV genotype) were 0.55 (95% CI: 0.29 to 0.99; P=0.037) and 0.80 (95% CI: 0.48 to 1.32; P=0.36), respectively (Table 2).

| TABLE 1. General Characteristics and Selected Risk Factors for Carotid Artery Occlusive Disease |
|-----------------|-----------------|-----------------|-----------------|
| Age             | 68±6            | 65±7            | 0.02            |
| Men:women       | 78:38           | 133:71          | 1.09 (0.65–1.83)| 0.71            |
| Hypertension    | 68/106          | 64%             | 3.13 (1.86–5.28)| 0.001           |
| Cigarette smoking| 78/104          | 75%             | 2.36 (1.36–4.13)| 0.001           |
| Diabetes mellitus| 25/106          | 23%             | 1.60 (0.85–3.02)| 0.10            |
| Hypercholesterolemia| 80/102         | 78%             | 1.95 (1.09–3.58)| 0.016           |
TABLE 2. Prevalence of Genotypes and Allele Frequencies in Patients and Controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (108)</th>
<th>Controls (204)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>V249I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V/V</td>
<td>63</td>
<td>108</td>
<td>0.53 (0.30–1.96)</td>
<td>0.56</td>
</tr>
<tr>
<td>V/I</td>
<td>34</td>
<td>84</td>
<td>0.78 (0.30–1.96)</td>
<td>0.56</td>
</tr>
<tr>
<td>I/I</td>
<td>11</td>
<td>12</td>
<td>0.53 (0.22–1.24)</td>
<td>0.11</td>
</tr>
<tr>
<td>T280M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>87</td>
<td>142</td>
<td>0.78 (0.30–1.96)</td>
<td>0.56</td>
</tr>
<tr>
<td>T/M</td>
<td>18</td>
<td>58</td>
<td>0.53 (0.22–1.24)</td>
<td>0.11</td>
</tr>
<tr>
<td>M/M</td>
<td>3</td>
<td>4</td>
<td>0.78 (0.30–1.96)</td>
<td>0.56</td>
</tr>
<tr>
<td>M allele frequency</td>
<td>0.11</td>
<td>0.16</td>
<td>0.66 (0.38–1.12)</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Odds ratios for genotypes were calculated with T/M+M/M vs T/T and V/I+I/I vs V/V.

 Nineteen patients had had TIA, 30 had stroke or silent cerebral infarction, and 59 were asymptomatic.

 No differences in clinical signs and symptoms (eg, TIA's and stroke or silent cerebral infarction) were found in correlation with T280M and V249I. CX3CR1 I249 allele was significantly associated with hard plaques (OR: 0.38; 95% CI: 0.13 to 1.05; P=0.037) (Table 3). When haplotypes were considered, no correlation was found with both presence of plaque and clinical signs (Table 4). Multiple logistic regression analysis was performed to identify possible independent risk factors for ICA occlusive disease among patients and controls. The following variables were considered: hypertension, cigarette smoking, diabetes mellitus, hypercholesterolemia, and I249 and M280 allele. Hypercholesterolemia and diabetes were not found to be an independent risk factor for ICA occlusive disease in our series as well as the presence of the I249 allele. All the other 3 variables considered were found to be independent risk factors: hypertension (P=0.001; OR: 2.75; 95% CI: 1.64 to 4.61), cigarette smoking (P=0.018; OR: 2.40; 95% CI: 1.27 to 4.54), and M280 allele presence (P=0.043; OR: 0.47; 95% CI: 0.22 to 0.97) (Table 5). No significant correlations were observed in the subgroup affected from coronary artery disease (data not shown).

TABLE 4. Combined Genotype Frequencies of the V249I and T280M Polymorphisms of the CX3CR1 in Cases and Controls

<table>
<thead>
<tr>
<th>Combined Genotype</th>
<th>V249I</th>
<th>T280M</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>V/V</td>
<td>TT</td>
<td>63 (58)</td>
<td>104 (51)</td>
</tr>
<tr>
<td>2</td>
<td>I/I</td>
<td>TT</td>
<td>4 (4)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>3</td>
<td>T/T</td>
<td>MM</td>
<td>3 (3)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>4</td>
<td>I/I</td>
<td>TM</td>
<td>4 (4)</td>
<td>6 (3)</td>
</tr>
<tr>
<td>5</td>
<td>T/T</td>
<td>TT</td>
<td>20 (18)</td>
<td>36 (18)</td>
</tr>
<tr>
<td>6</td>
<td>I/I</td>
<td>TM</td>
<td>14 (13)</td>
<td>52 (25)</td>
</tr>
</tbody>
</table>

Combined genotypes not reported in this Table were not found in our series. Numbers in brackets express percentages.

Discussion

We have shown that CX3CR1 M280, a mutant form of the chemokine receptor CX3CR1, that is defective in adhesive and chemotactic activity, is associated with lower risk of ICA occlusive disease and that the I249 is associated with hard carotid plaques, which seem to be more stable than soft ones.

Recruitment of circulating monocytes to the arterial intima contributes to the formation of atherosclerotic lesions and may participate in their destabilization. Leukocyte emigration from blood into tissue is mediated by multiple adhesion molecules and chemokines, which orchestrate specific steps of emigration and regulate peripheral recruitment of different leukocytes depending on their expression patterns of chemokine receptors.

The expression of a less efficient CX3CR1 receptor on PBMCs reduces the ability of endothelial membrane-bound FKN to attract monocytes, resulting in a less efficient mechanism of adhesion and, theoretically, in a reduced foam cell content in the atherosclerotic plaque. This could contribute to plaque stability.

The V249I and T280M polymorphisms, located in the sixth and seventh transmembrane domains of the CX3CR1 protein, respectively, may affect the risk of acute coronary disease. Moatti et al in their study about FKN binding-demonstrated that FKN binding-site density on PBMCs from individuals carrying either VI-TT or VI-TM genotypes was ~40% lower than on PBMCs from individuals bearing the reference genotype VV-TT. This would be expected to reduce monocyte adhesion to injured endothelium; therefore, this is a potential mechanism for the reduced risk of acute coronary events associated with this genotype. In our study, the presence of T280 allele reduced by 2-fold the risk of ICA...
occlusive disease independent of established ICA occlusive disease risk factors (patients with TM+MM genotypes versus TT, OR: 0.47; 95% CI: 0.22 to 0.97; P = 0.037).

Stroke is the third leading cause of death in the Western world and a major cause of disability in adults. It is well known that ICA atherosclerotic occlusive disease predisposes to cerebral events, and histology of atherosclerosis is well defined. Soft carotid plaques, otherwise defined as unstable or vulnerable, are characterized by increased accumulation of peripheral blood mononuclear inflammatory cells, particularly macrophages and T lymphocytes, and by a large lipid core covered by a fibrous cap.25 Hunt et al have recently demonstrated that patients with calcification of carotid plaques (hard or stable plaques) had fewer symptoms of stroke and transient ischemic attack than those without calcification.26

The I249 allele presence in our study correlates well with the presence of calcification in the plaques (OR: 0.38; 95% CI: 0.13 to 1.05; P = 0.037). In our study, the presence of I249 does not significantly correlate with reduction in stroke or transient ischemic attack, albeit only 32% (6 of 17) of patients with soft plaques and I249 allele had cerebral symptoms, compared with 53% (19 of 36) of patients with the same histologic type of plaques and V249 homozygosity (data not shown). The small number of cases could justify in part the loss of correlation between clinical signs and CX3CR1 polymorphisms. Another observation should be made to explain this lack of statistical correlation: the target of the treatment (medical as well as surgical) of carotid occlusive disease independent of established ICA occlusive disease risk factors (patients with TM/H11001 genotype versus MM genotypes) versus I249 homozygosity (data not shown). The small number of cases could justify in part the loss of correlation between clinical signs and CX3CR1 polymorphisms. Another observation should be made to explain this lack of statistical correlation: the target of the treatment (medical as well as surgical) of carotid occlusive disease independent of established ICA occlusive disease risk factors (patients with TM/H11001 genotype versus MM genotypes); 249 homozygosity (data not shown).

In conclusion, our results, for the first time to our knowledge, correlate the CX3CR1 polymorphisms with the pathogenesis and the progression of ICA occlusive disease. Although suggestive, these results need confirmation in further prospective cross-sectional studies.

References

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