Smooth Muscle Cells in Human Atherosclerotic Plaques Express the Fractalkine Receptor CX3CR1 and Undergo Chemotaxis to the CX3C Chemokine Fractalkine (CX3CL1)

Andrew D. Lucas, PhD*; Christina Bursill, PhD*; Tomasz J. Guzik, MD, PhD; Jerzy Sadowski, MD, PhD; Keith M. Channon, MD; David R. Greaves, PhD

Background—Chemokines are important mediators of inflammatory cell recruitment that play a significant role in atherosclerosis. Fractalkine (CX3CL1) is an unusual membrane-bound chemokine that mediates chemotaxis through the CX3CR1 receptor. Recently, functional polymorphisms in the human CX3CR1 gene have been described that are associated with coronary artery disease.

Methods and Results—We investigated the expression of the CX3C chemokine fractalkine and its receptor CX3CR1 in human coronary artery plaques by immunocytochemistry. We show that a subset of mononuclear cells expresses high levels of fractalkine in human coronary atherosclerotic plaques and that smooth muscle cells within the neointima express the fractalkine receptor CX3CR1. There is a positive correlation between the number of fractalkine-expressing cells and the number of CX3CR1-positive cells in human atherosclerotic plaques (r = 0.70, n = 15 plaques). Furthermore, we demonstrate that cultured vascular smooth muscle cells express the CX3CR1 receptor and undergo chemotaxis to fractalkine that can be inhibited by G protein inactivation by pertussis toxin.

Conclusions—These results suggest that in human atherosclerosis, fractalkine, rather than mediating inflammatory cell recruitment, can act as a mediator of smooth muscle cell migration. (Circulation. 2003;108:2498-2504.)

Key Words: chemokines | atherosclerosis | macrophages | muscle, smooth | remodeling

Local and systemic inflammation play critical roles in the development of atherosclerosis.1–4 Monocytes that adhere to inflamed endothelium are recruited into the subendothelial space, where they differentiate into macrophages. Recruited macrophages endocytose modified forms of LDL via scavenger receptors to form foam cells, which are the predominant cell type present in fatty streak lesions.5 These lesions develop into fibrofatty plaques, which contain large numbers of macrophages and CD4+ T cells and show evidence of smooth muscle cell (SMC) migration and proliferation.5–7

Chemokines are a superfamily of chemoattractant cytokines that play an essential role in leukocyte recruitment in acute and chronic inflammation.5,8–10 A key role for the CC chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2) has been demonstrated in murine models of atherosclerosis. MCP-1 or the CCR2 gene exhibit reduced atherosclerotic lesions in mouse and apoE knockout animals.16,18,19

Fractalkine (CX3CL1) is synthesized as a membrane-bound molecule with the chemokine motif attached to a long mucin stalk.20 It is expressed by a range of cell types in human atherosclerotic plaques, including macrophages and SMCs.15 Other CC chemokines that are expressed in human atherosclerotic plaques include eotaxin (CCL11), macrophage-derived chemokine (MDC/CCL22), and thymus- and activation-regulated chemokine (TARC/CCL17).16,17 A potential role for CXC chemokines in the development of atherosclerotic lesions has also been demonstrated in gene knockout animals.16,18,19

Fractalkine (CX3CL1) is synthesized as a membrane-bound molecule with the chemokine motif attached to a long mucin stalk.20 It is expressed by a range of cell types in vivo, including neurons,21 epithelial cells,22 and macrophages.17 The receptor for fractalkine, CX3CR1, is a G protein–coupled receptor (GPCR) with 7 transmembrane (TM7)–spanning regions.23 The CX3CR1 receptor is able to mediate tight adhesion of cells to immobilized forms of fractalkine under flow conditions in vitro.24,25 Fractalkine gene knockout mice have no obvious defects in leukocyte trafficking but are less susceptible to cerebral ischemia-reperfusion injury.26,27 Further evidence for a possible role of fractalkine in cardiovascular disease has come from recent observations in human subjects suggesting that a single amino
acid polymorphism at position 249 of the CX₃CR1 receptor is an independent risk factor for coronary artery disease²⁸,²⁹ (reviewed by Alexander³⁰). However, the biological rationale for these observations remains unclear.

Accordingly, we investigated the presence of fractalkine and its cognate receptor, CX₃CR1, in human coronary artery atherosclerotic plaques. We show that fractalkine expression is principally by mononuclear cells, whereas the fractalkine receptor CX₃CR1 is expressed by intimal SMCs. Furthermore, we demonstrate that human SMCs can undergo chemotaxis to fractalkine in vitro. These results lead us to propose fractalkine as a newly identified mediator of SMC migration in human coronary atherosclerosis.

### Methods

#### Human Tissues

Sections of right and left coronary arteries were taken from hearts removed from transplant recipients (9 men, 1 woman; mean age, 51 years) with appropriate local ethical approval and written informed consent. For more details see Table 1.

#### Human Primary Cells and Cell Lines

Human coronary artery SMCs were prepared from a 21-year-old male donor (TCS CellWorks). Cells were typically passaged 2 times before use. For immunohistochemistry experiments, 1·10⁶ cells were seeded into glass chamber slides (Nunc) and grown until confluent. For Western blot analysis, cells (5·10⁴) were seeded in 6-well cluster dishes (Costar) and grown until confluent. For immunohistochemistry experiments, sections were stained with Topro blue. Migration was quantified as total pixel count of blue cells under the confocal microscope (scans performed in triplicate, 3 scans/membrane). Signal transduction via G protein-coupled receptors was blocked by preincubating cells with pertussis toxin (PTX, 250 ng/mL, Sigma-Aldrich) for 2 hours before chemotaxis.

#### Chemotaxis Assays

Cell migration was assessed by use of transwell membranes (ChemtoX, 6-mm diameter, 8-µm pore size) as described previously.³² Cells were harvested, placed on transwell membranes, and allowed to migrate toward increasing concentrations of fractalkine or platelet-derived growth factor (PDGF)-AB. Migrated cells on the underside of membranes were fixed (paraformaldehyde-sucrose) and stained with Topro blue. Migration was quantified as total pixel count of blue cells under the confocal microscope. Western blot analysis was performed as described previously.²² Detergent lysates derived from human peripheral blood mononuclear cells (PBMCs) or CD14⁺ monocytes were used as a positive control.

#### Immunohistochemistry

The following primary antibodies were used: rat anti-human CX₃CR1 (isotype IgG2b, clone 2A9-1, MBL); rabbit anti-human CX₃CR1 N-terminal peptide (ProSci Inc); goat anti-fractalkine chemokine domain (R&D Systems); mouse anti-hemagglutinin epitope (YPYDVPDYA) (isotype IgG1, clone 16B12, Covance); mouse anti-human CD14 (isotype IgG2a, clone TUK4, Dako AS), mouse anti-human CD3 (isotype IgG1, clone UCHT1, Dako), and mouse anti-human CD68 (isotype IgG1, Clone EBM11, Dako). All antibodies were diluted in PBS containing 1% BSA and 0.2% Triton X-100, and sections were incubated with primary antibodies overnight at 4°C. In cases in which a green label was required for visualization, samples were incubated with anti–species-specific IgG conjugated to Alexa Fluor 568 (Molecular Probes) for 1 hour at 4°C. In cases in which a green label was required for visualization, samples were incubated with anti–species-specific IgG conjugated to biotin (Dako) for 1 hour at 4°C. Finally, a streptavidin–fluorescein isothiocyanate (HTIC) reagent (Amersham) was applied for 30 minutes at 4°C. Double immunofluorescent labeling involved sequential single-labeling procedures, with all incubations at 4°C. Immunohistochemical detection of single antigens was performed as described previously.²⁷,²²

### Table 1. Coronary Artery Samples Used in This Study

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Donor Sex</th>
<th>Donor Age, y</th>
<th>Preexisting CAD</th>
<th>Lesion Morphology</th>
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<tbody>
<tr>
<td>HCA007L</td>
<td>M</td>
<td>50</td>
<td>Yes</td>
<td>Thin fibrous cap atheroma</td>
</tr>
<tr>
<td>HCA010R</td>
<td>M</td>
<td>42</td>
<td>Yes</td>
<td>Intimal thickening</td>
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<tr>
<td>HCA011R</td>
<td>M</td>
<td>45</td>
<td>Yes</td>
<td>Intimal thickening</td>
</tr>
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<td>HCA013R</td>
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<td>57</td>
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<tr>
<td>HCA014R</td>
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<td>Intimal thickening</td>
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<tr>
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<tr>
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<td>55</td>
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<td>Fibrous cap atheroma with hemorrhage</td>
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<tr>
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<td>55</td>
<td>Yes</td>
<td>Thin fibrous cap atheroma</td>
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<tr>
<td>HCA022R</td>
<td>M</td>
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<td>Yes</td>
<td>Fibrous cap atheroma with hemorrhage</td>
</tr>
<tr>
<td>HCA022L</td>
<td>M</td>
<td>49</td>
<td>Yes</td>
<td>Fibrous cap atheroma with hemorrhage</td>
</tr>
</tbody>
</table>

Sample numbers refer to coronary arteries obtained from different patients (9 male, 1 female) undergoing heart transplantation. Six patients showed preexisting coronary artery disease (CAD) detected by angiography, 6 had a previous myocardial infarction, and 2 had undergone CABG. Four patients exhibited dilated cardiomyopathy, 7 were hypertensive, and 2 had diabetes mellitus. L and R indicate left and right coronary artery, respectively.

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Validation of Anti-CX₃CR1 Antisera

To verify the specificity of a range of anti-CX₃CR1 reagents, we transfected HEK-293 cells with an expression vector encoding CX₃CR1 tagged at the C-terminus with the influenza hemagglutinin (HA) epitope tag. FACS analysis of transfected cells with mouse anti-hemagglutinin monoclonal antibody detected a strongly positive population of transfected cells, and a similar population was revealed by use of a rat anti-human CX₃CR1 monoclonal antibody. Two commercial rabbit polyclonal reagents varied in their ability to detect CX₃CR1 antigens in FACS and immunofluorescence, with the ProScience anti-N-terminal peptide reagent giving specific staining of CX₃CR1-transfected CHO cells (data not shown).

Results

Expression of Fractalkine and CX₃CR1 in Human Coronary Atherosclerosis

To address whether fractalkine could be detected in association with coronary artery disease, freshly isolated human coronary artery segments from heart transplant recipients were examined by immunohistochemistry. We examined 19 separate arteries sampled from 10 patients (Table 1). Most but not all arteries showed intimal areas characterized by an infiltrate of mononuclear cells, clearly counterstained with hematoxylin in the goat IgG isotype control experiment (Figure 1A). A proportion of the infiltrating mononuclear cells within the intima stained strongly positive for fractalkine (Figure 1B). Consistent with our previous studies on carotid and femoral arteries,²⁰ we did not detect fractalkine immunostaining of luminal endothelial cells in any of the sections studied. Indeed, occasional fractalkine-positive mononuclear cells were seen adhering to the unstained luminal endothelium that showed clear staining for von Willebrand factor in adjacent sections (data not shown).

Next, we performed double immunofluorescence labeling to identify the fractalkine-positive cells within coronary artery sections (red-staining cells in Figure 2, A–E, summarized in Table 2). Infiltrating CD3-positive T cells (green; Figure 2A) were present in most lesions examined (9 of 10 plaques), whereas CD14 monocytes (green in Figure 2C) were detected in 4 of 6 lesions examined and were only rarely double positive for fractalkine expression. These CD14 fractalkine double-positive cells were located just beneath the luminal endothelium, consistent with their being recently
transmigrated monocytes (Figure 2C). A more substantial proportion of the fractalkine-positive cells double-stained for the CD68 antigen (stained yellow in Figure 2B; CD68 single-positive cells are green). In lesions containing large CD68-positive cells with the appearance of foam cells, there was a lack of double labeling with fractalkine (data not shown). Smooth muscle actin–positive cells were present within the intima (stained green in Figure 2D), and in 7 of 14 lesions examined, we were able to detect some SMCs that expressed fractalkine.

Thus, cells expressing fractalkine in human coronary artery plaques included CD68-positive macrophages, CD14-positive monocytes, some SMCs, and a significant population of mononuclear cells that were negative for these markers but not endothelial cells.

To determine the relationships between cells expressing fractalkine and those expressing the fractalkine receptor in human coronary artery plaques, we performed double immunofluorescent staining for the fractalkine receptor CX3CR1. Strikingly, fractalkine receptor–positive cells with the characteristics of SMCs (green in Figure 2E) were present adjacent to fractalkine-positive cells (red in Figure 2E) within the intimal region in 16 of the 19 coronary lesions examined, and in 8 of 19 lesions examined, we detected some cells that were positive for both fractalkine and CX3CR1. Confirmation of the CX3CR1-positive cells as SMCs was obtained by double staining with an anti–smooth muscle actin antibody (yellow cells in Figure 2F). The numbers of fractalkine– and SMC actin–positive cells were determined, no relationship could be demonstrated (Figure 3A; \( r = 0.1 \), \( P = \text{NS} \)). However, there was a strong positive correlation between the numbers of fractalkine-positive and fractalkine receptor–positive cells within lesions (Figure 3B; \( r = 0.70 \), \( P < 0.01 \)).

**Primary Human SMCs Express the Fractalkine Receptor CX3CR1 and Undergo Chemotaxis to Fractalkine**

To further investigate expression of the fractalkine receptor CX3CR1 by SMCs, we cultured primary human coronary artery SMCs to a 60% to 70% confluence on glass chamber slides. Cells were fixed and stained for CX3CR1 and smooth muscle actin expression. We detected strong, punctate cellular staining for the fractalkine receptor CX3CR1 relative to rat IgG2b isotype control antibody (Figure 4A). This staining was both perinuclear and clearly distributed on the plasma membrane (Figure 4A, arrow). Western blotting of primary human PBMCs, CD14 positively selected monocytes, and primary human coronary artery SMC lysates for CX3CR1 revealed a band of 45 kDa in all 3 cell preparations (Figure 4B). Human primary SMC lysates but not monocyte lysates were positive for smooth muscle actin expression (Figure 4C). These observations confirm that primary human SMCs express the fractalkine receptor CX3CR1.
To investigate the responsiveness of human SMCs to recombinant human fractalkine, we used the human SMC line HCM-601EB and primary SMCs in chemotaxis assays in modified Boyden chambers. HCM-601EB cells migrated in response to both recombinant fractalkine (Figure 5A) and PDGF-AB (Figure 5B), showing a characteristic “bell-shaped” curve typical of chemokine migration assays. The maximal migration occurred to 25 nmol/L fractalkine and 4 nmol/L PDGF-AB. As a further test of specificity, we preincubated HCM-601EB cells with PTX (250 ng/mL), an inhibitor of G protein-coupled receptor signaling. As expected, the migratory response to fractalkine was blocked by PTX (Figure 5C), because the fractalkine receptor is a Gi-coupled receptor. In contrast, the response to PDGF, mediated by the tyrosine kinase PDGF receptor, was not affected (Fig 5D). Importantly, we demonstrated that primary human coronary artery SMCs undergo chemotaxis to fractalkine (Figure 5E) and that this chemotaxis is sensitive to PTX inhibition (Figure 5F).

**Discussion**

We have undertaken a detailed analysis of the expression pattern of the CX3C chemokine fractalkine and its receptor, CX3CR1, in human coronary atherosclerosis. Fractalkine is expressed at a high level by a subset of inflammatory cells but not by endothelial cells. The number of fractalkine-expressing cells in the intima is directly correlated with the presence of CX3CR1+ cells, most of which are SMCs. Furthermore, we have demonstrated that primary human SMCs express the CX3CR1 receptor and can undergo specific CX3CR1-mediated chemotaxis to fractalkine in vitro. Our results suggest that fractalkine, rather than acting as a proinflammatory cytokine in human atherosclerosis, may play an important role in vascular remodeling through the recruitment of SMCs into the atherosclerotic plaque.

In vitro SMCs cultured endothelial cells treated with a range of inflammatory mediators express fractalkine mRNA and protein. However, we saw no evidence of...
endothelial expression of fractalkine in human coronary atherosclerosis (Figure 1). Rather, infiltrating mononuclear cells present in the intima of human coronary artery atherosclerotic plaques express high levels of fractalkine. These cells are primarily CD14- and CD68-negative and may represent recruited monocytes that are in the process of differentiation. We used validated anti-CX3CR1 antibodies to identify fractalkine receptor–expressing cells in human atherosclerosis. Quantitative image analysis shows a positive correlation between the presence of CX3CR1+ cells in the neointima and fractalkine-expressing mononuclear cells (Figure 3B). The majority of these CX3CR1+ cells were shown by double immunofluorescence to be SMCs. Taken together with the chemotaxis data presented in Figure 5, our observations strongly suggest that rather than playing a role in continuing inflammatory cell recruitment, fractalkine expression within human atherosclerotic plaques may be acting to recruit SMCs. By recruiting cells capable of synthesizing substantial amounts of extracellular matrix, fractalkine expression may favor the development of stable atherosclerotic lesions rather than macrophage-rich vulnerable plaques. It will be interesting to determine whether developing atherosclerotic plaques that express high levels of fractalkine within the neointima progress to stable plaques with high numbers of SMCs and fibrous caps. In a recent article, Chandrasekar et al.14 reported CX3CR1-dependent proliferation of primary rat aortic SMCs in response to fractalkine. However, an overen-thusiasmonic recruitment of SMCs into a developing plaque followed by fractalkine-driven SMC proliferation could result in SMC-dominated intimal hyperplasia.

Recently, coding region polymorphisms, V249I and T280M, have been identified in the human CX3CR1 gene and appear to be associated with reduced risk of developing coronary artery disease.28,29,35 PBMCs prepared from human donors with different CX3CR1 genotypes exhibit differences in the number of fractalkine-binding sites per cell, and cells transfected with variant receptor show impaired ligand-dependent cell adhesion.35 However, the biological rationale for an association between genetic variation in CX3CR1 and coronary artery disease has remained unclear. Our study now provides novel data on CX3CR1 expression in human coronary atherosclerosis. Our observations suggest that a potential mechanistic link between CX3CR1 gene polymorphisms in human populations and the development of coronary artery disease may be exerted not only at the level of monocyte recruitment but also at the level of atherosclerotic plaque stability through modulation of SMC recruitment. The only other identified high-affinity receptor for fractalkine is encoded by the US28 open reading frame of human cytomegalovirus, and evidence has been presented for a link between cytomegalovirus infection and SMC proliferation in atherosclerosis.36,37

More than 40 different human chemokines have been described, and the majority of CC chemokines have been shown to be potent mediators of either monocyte or T-cell chemotaxis.10 Indeed, CX3CR1+ apoE double-knockout mice exhibit reduced macrophage recruitment into atherosclerotic lesions.38,39 However, our identification of fractalkine as a chemoattractant for SMCs suggests that this unusual CX3C chemokine merits further investigation in human atherosclerosis and other forms of vascular remodeling, including restenosis and arteriosclerosis.

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References


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