Linked Chromosome 16q13 Chemokines, Macrophage-Derived Chemokine, Fractalkine, and Thymus- and Activation-Regulated Chemokine, Are Expressed in Human Atherosclerotic Lesions

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Abstract—Chemokines are important mediators of macrophage and T-cell recruitment in a number of inflammatory pathologies, and chemokines expressed in atherosclerotic lesions may play an important role in mononuclear cell recruitment and macrophage differentiation. We have analyzed the expression of the linked chromosome 16q13 genes that encode macrophage-derived chemokine (MDC/CCL22), thymus- and activation-regulated chemokine (TARC/CCL17), and the CX3C chemokine fractalkine (CX3CL1) in primary macrophages and human atherosclerotic lesions by reverse transcription–polymerase chain reaction and immunohistochemistry. We show that macrophage expression of the chemokines MDC, fractalkine, and TARC is upregulated by treatment with the Th2-type cytokines interleukin-4 and interleukin-13. High levels of MDC, TARC, and fractalkine mRNA expression are seen in some, but not all, human arteries with advanced atherosclerotic lesions. Immunohistochemistry shows that MDC, fractalkine, and TARC are expressed by a subset of macrophages within regions of plaques that contain plaque microvessels. We conclude that MDC, fractalkine, and TARC, which are chromosome 16q13 chemokines, could play a role in mononuclear cell recruitment into atherosclerotic lesions and influence the subsequent inflammatory response. Macrophage-expressed chemokines upregulated by interleukin-4 may be useful surrogate markers for the presence of Th2-type immune responses in human atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2001;21:923-929.)

Key Words: chemokines ■ atherosclerosis ■ macrophages ■ Th2-type T cells

Atherosclerosis is a major cause of morbidity and mortality in western countries. Atherosclerotic lesions within major blood vessels are responsible for myocardial infarction, strokes, and peripheral vascular disease. The initial event in the development of the atherosclerotic lesion is the adhesion of monocytes to the endothelial cells of the blood vessel wall and the extravasation of monocytes into the subendothelial space. Recruited monocytes differentiate into macrophages and accumulate modified forms of LDL via scavenger receptors to yield lipid-laden foam cells. Chronic recruitment of mononuclear cells into the developing lesion contributes to the growth of lesions and occlusion of the blood vessel. Macrophages and T cells within atherosclerotic lesions produce an array of cytokines, growth factors, and inflammatory mediators, which are likely to play a role in continued mononuclear cell recruitment.

Chemoattractant cytokines (chemokines) are small disulfide-linked polypeptides of typically 60 to 70 amino acids in length that are potent chemoattractants for leukocytes. The chemokine supergene family, which was unrecognized 10 years ago, now has >40 different members classified into different subfamilies on the basis of conserved structural features. The CXC (or α) chemokines have a single amino acid separating the first 2 cysteine residues of the protein, whereas CC (or β) chemokines have no amino acid separating the signature C1 and C2 cysteine residues. The CC chemokine monocyte chemotactic protein-1 (MCP-1/CCL2), a potent chemoattractant for monocytes, has been shown to be expressed in human atherosclerotic lesions. Mice homozygous for a targeted deletion of the MCP-1 gene or the gene that encodes the MCP-1 receptor CCR2 exhibit a reduced incidence of atherosclerotic lesions when they are fed a high-fat diet. The involvement of chemokines other than MCP-1 in the pathogenesis of atherosclerosis is suggested by the observation that lethally irradiated LDL receptor–knockout mice repopulated with bone marrow from CXCR2-
deficient mice had smaller aortic lesions with reduced numbers of recruited monocytes.\textsuperscript{14}

Fractalkine/CX3CL1 is a novel chemokine that differs from other chemokines in that it has 3 intervening amino acids between the 2 cysteine residues of the chemokine motif (CX3C) and exists as a membrane-bound molecule with the chemokine motif attached to a long mucin stalk.\textsuperscript{15} When cleaved from the cell surface, soluble forms of fractalkine mediate the chemotaxis of monocytes and T cells.\textsuperscript{15} Mobilized forms of fractalkine have been shown to mediate tight adhesion of cells carrying the CX3CRI receptor, and this adhesion does not require integrins, calcium, or an opposing cell membrane.\textsuperscript{16} Furthermore, fractalkine-dependent firm adhesion of monocytes and T cells can occur under flow conditions.\textsuperscript{17}

We have analyzed the expression of macrophage-derived chemokine (MDC), fractalkine, and thymus- and activation-regulated chemokine (TARC), which are linked chromosome 16q13 chemokines, in primary human macrophages and in atherosclerotic lesions of human arteries. Macrophage expression of MDC and TARC is upregulated by the Th2 cytokines interleukin (IL)-4 and IL-13 but not by IL-10 or the Th1 cytokine interferon-\(\gamma\) (IFN-\(\gamma\)). The chemokines MDC, fractalkine, and TARC are expressed by a subset of macrophages within human atherosclerotic plaques associated with intraplaque microvessels in regions of neovascularization.

**Methods**

**Human Tissues**

Aortic tissue samples used for RNA preparations were obtained from transplant organ donors (10 females and 10 males, aged 16 to 66 years), and carotid tissue samples were obtained from patients undergoing endarterectomy (2 females and 8 males, aged 66 to 82 years). The use of discarded human tissue for research purposes was approved by the Ethics Committee of Addenbrooke’s Hospital, Cambridge. The arterial samples used for immunohistochemistry were obtained from patients undergoing surgical procedures at the John Radcliffe Hospital, Oxford. The use of discarded human tissue for research purposes was approved by the Central Oxford Research Ethics Committee.

**Human Primary Cell Culture**

Macrophages were prepared from peripheral blood mononuclear cells (PBMCs) by adhesion. Briefly, PBMCs prepared from buffy coats by Ficoll centrifugation were incubated in a 225-cm\(^2\) tissue culture flask for 3 hours and washed once with PBS to eliminate unattached cells. After 3 days in culture, serum-free medium (X-Vivo-10, Bio-Whittaker) containing IFN-\(\gamma\), IL-4, IL-10, or IL-13 (69 ng/mL, R&D Systems) was added, and this was repeated on day 7. The resultant cDNA preparations were used as templates in 20 \(\mu\)L RT–polymerase chain reaction (PCR) reactions containing 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 1.5 mmol/L MgCl\(_2\), 0.1% Triton X-100, 0.2 mmol/L each dNTP, 250 ng each primer, and 2.5 \(\mu\)L Taq Polymerase (Promega). The following primers were used for RT-PCR reactions: fractalkine, 5‘-ATGACATCAAAGATAC-CTGTAGC and 5‘-AGGAATCATCGAAGAAGTGTC (MDC); fractalkine, 5‘-ACAGACTGACCTCTTGGTTG and 5‘-GCTCAGTATT-GAGAATCATC (TARC), 5‘-GGACCTGACACAGAGAGAGAGACTC and 5‘-GGTACACGTCTTCACGTTT (CD68), 5‘-GAGGCTCTGACCATCTCCTGTA and 5‘-CGAGTTGCTGT-CACTGGAAGT; and \(\beta\)-microglobulin, 5‘-GATTCAGGTT -TACTCAGC and 5‘-CCATGATGCTGTTACATG. RT-PCR reactions were performed for 30 amplification cycles (94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds), except for \(\beta\)-microglobulin and CD68 reactions, which were amplified for 25 cycles. Half of each RT-PCR reaction was reamplified on a 1% agarose gel and transferred to Hybond NX nylon membranes (Amersham) by Southern blotting. Filters were hybridized with \(\alpha\)-\(\beta\)dCTP–labeled probe fragments, and the amount of amplified product in each sample was measured by using electronic autoradiography (Instant Imager, Packard). Serial dilutions of cDNA samples were analyzed to show that the RT-PCR signal was directly proportional to the amount of cDNA template.

**Immunohistochemistry**

The preparation of an anti–fractalkine-specific antiserum with use of a synthetic peptide corresponding to amino acids 352 to 370 of the intracellular domain of the fractalkine has been described previously.\textsuperscript{18} An affinity-purified anti–amino terminal fractalkine rabbit antiserum\textsuperscript{15} was a gift of Dr Thomas Schall (ChemoCentryx Inc, San Carlos, Calif.). Serial paraffin-embedded sections (7 to 10 \(\mu\)m) and frozen sections (7 \(\mu\)m) were stained for fractalkine and TARC expression. For RNA in situ hybridization, sections were counterstained with hematoxylin. Irrelevant class- and species-matched immunoglobulins were used as controls.

**Results**

**Localization of the MDC, Fractalkine, and TARC Genes to Chromosome 16q13**

We have reported previously that the human fractalkine gene is mapped to chromosome 16 by analysis of somatic cell hybrids.\textsuperscript{15} We isolated 3 cosmids and a PAC clone from chromosome 16 genomic DNA libraries with the use of fractalkine cDNA probes (Figure 924 Arterioscler Thromb Vasc Biol. June 2001

**RNA Preparation and RT-PCR**

Arterial tissue samples were dispersed by collagenase and elastase digestion, and cells were lysed in 150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L MgCl\(_2\), and 0.5% Nonidet P-40. Nuclei were pelleted by centrifugation, and the supernatant was adjusted to 1.5% SDS, extracted twice with Tris-buffered phenol, and ethanol-precipitated. Precipitated RNA was digested with RNase-free DNase I (Promega). Total RNA (5 \(\mu\)g) was used as a template in a 60-minute reverse transcription (RT) reaction at 42°C by using oligo dT\(_{12-18}\) primer and avian myeloblastosis virus reverse transcriptase. The resultant cDNA preparations were used as templates in 20 \(\mu\)L RT–polymerase chain reaction (PCR) reactions containing 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 1.5 mmol/L MgCl\(_2\), 0.1% Triton X-100, 0.2 mmol/L each dNTP, 250 ng each primer, and 2.5 \(\mu\)L Taq Polymerase (Promega). The following primers were used for RT-PCR reactions: fractalkine, 5‘-ATGACATCAAAGATAC-CTGTAGC and 5‘-AGGAATCATCGAAGAAGTGTC (MDC); fractalkine, 5‘-ACAGACTGACCTCTTGGTTG and 5‘-GCTCAGTATT-GAGAATCATC (TARC), 5‘-GGACCTGACACAGAGAGAGAGACTC and 5‘-GGTACACGTCTTCACGTTT (CD68), 5‘-GAGGCTCTGACCATCTCCTGTA and 5‘-CGAGTTGCTGT-CACTGGAAGT; and \(\beta\)-microglobulin, 5‘-GATTCAGGTT -TACTCAGC and 5‘-CCATGATGCTGTTACATG. RT-PCR reactions were performed for 30 amplification cycles (94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds), except for \(\beta\)-microglobulin and CD68 reactions, which were amplified for 25 cycles. Half of each RT-PCR reaction was reamplified for 1% agarose gel and transferred to Hybond NX nylon membranes (Amersham) by Southern blotting. Filters were hybridized with \(\alpha\)-\(\beta\)dCTP–labeled probe fragments, and the amount of amplified product in each sample was measured by using electronic autoradiography (Instant Imager, Packard). Serial dilutions of cDNA samples were analyzed to show that the RT-PCR signal was directly proportional to the amount of cDNA template.
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1A). Cosmid c325D6 was labeled with biotin and hybridized to G-banded metaphase spreads in fluorescent in situ hybridization experiments that localized the fractalkine gene to chromosome 16q13 (data not shown). Analysis of the GeneBridge IV radiation hybrid panel with fractalkine-specific PCR primers mapped the fractalkine gene between the framework markers D16S408 and D16S503 (data can be accessed online at http://sunny.ebi.ac.uk/RHdb/, accession No. RH86440). The MDC and TARC genes have been mapped to this same region of chromosome 16.19 Analysis of the fractalkine gene containing cosmids and the PAC clone by use of long-distance PCR, Southern blotting, and DNA sequencing showed that the MDC, fractalkine, and TARC genes are physically linked and are in the same transcriptional orientation (Figure 1A).

Expression of Chromosome 16q13 Chemokines in Mononuclear Phagocytes

A cDNA clone encoding MDC was originally isolated from a macrophage cDNA library,20 and MDC expression by macrophages has been reported to be upregulated by bacterial endotoxin21 and treatment with Th2-type cytokines.22 By contrast, TARC has been reported to be a DC-specific marker.23 We prepared macrophages and DCs from PBMCs and analyzed the expression of the linked chromosome 16q13 chemokines by RT-PCR. MDC mRNA expression in primary macrophages is markedly induced by treatment with the Th2-type cytokine IL-4 (Figure 1B). Similar results were obtained with macrophages prepared from 4 different donors. Although the level of MDC expression in untreated macrophages varied between donors, all 4 donors showed a 5- to 10-fold increase in MDC mRNA expression on addition of IL-4. TARC mRNA is readily detectable in immature and mature macrophages and is induced by IL-4 treatment of primary human macrophages (Figure 1B). A similar pattern of expression is seen with the use of fractalkine-specific PCR primers, but the level of expression of fractalkine mRNA is lower than that for MDC or TARC. Supernatant medium was collected from IL-4–treated macrophages and analyzed by Western blotting. The data of Figure 1C show that secretion of TARC into the medium is completely dependent on the addition of IL-4; similar results were obtained with macrophages from 3 independent donors.

We analyzed MDC and TARC mRNA expression by human primary macrophages treated with other cytokines characteristic of Th2- and Th1-type immune responses. Figure 2 shows that macrophage expression of MDC and TARC mRNA is markedly upregulated by treatment with IL-4 and IL-13 but not by treatment with IL-10 or the Th1 cytokine IFN-γ. Cytokine-treated macrophages show similar levels of CD68 mRNA (Figure 2), and significant differences in gene expression in IL-10– and IFN-γ–treated macrophages were detected in differential display RT-PCR experiments (data not shown).

Fractalkine, MDC, and TARC mRNA Expression in Human Arteries

We undertook a semiquantitative RT-PCR analysis of fractalkine, MDC, and TARC expression in RNA prepared from a panel of 30 human aortic and carotid artery tissue samples, which had been selected for evidence of calcified plaques (C.

Figure 1. Chemokine expression in macrophages and DCs. A, Upper line shows arrangement of MDC, fractalkine, and TARC genes in the human genome. Black boxes represent coding regions, and white boxes represent untranslated regions and intervening sequences. Arrows above the gene symbols denote the transcriptional orientation of the genes. Relative positions of the chemokine genes were determined by long-distance PCR with use of PCR primers derived from the 5′ and 3′ regions of the MDC, fractalkine, and TARC genes, with the indicated recombinant cosmids and PAC clones used as templates.25 NotI restriction enzyme sites are denoted by the letter N, and NotI sites present in the cosmid cloning vector are shown by the letter N in parentheses. Dashed lines at both ends of the PAC clone indicate that the position of the ends of the clone insert have not been determined relative to the chemokine genes. The organization of the chemokine locus determined by analysis of cosmids and PAC clones is in agreement with the sequence data of BAC clone CT9875K-A-152ES deposited with GenBank (accession No. AC004382). B, Total RNA prepared from human macrophages (Møs) and DCs (imm. indicates immature; mat., mature) was used as a substrate for the generation of cDNA in the presence (+) or absence (−) of reverse transcriptase and PCR amplified with primers that span at least 1 intron in the indicated human genes. PCR products were analyzed by agarose gel electrophoresis. PCR reactions were performed for 30 cycles (MDC and TARC), 35 cycles (fractalkine [FK]), and 25 cycles (β2-microglobulin [β2M]). C, Supernatant cultures (30 μL = 15 μg) from primary Møs treated for 24, 48, or 72 hours in the presence (+) or absence (−) of IL-4 were fractionated by SDS PAGE, transferred to nylon membranes, and incubated with an antiserum that detects human MDC. A band that comigrates with recombinant human MDC at ∼10 kDa in IL-4–treated cell supernatants is indicated by the arrowhead. A nonspecific band of >30 kDa is detected in all samples with this antibody.

Shanahan, K., Tyson, P.L., Wessberg, unpublished data, 2001). RNA samples 1 through 10 were prepared from aortic samples that contained fatty streak lesions, samples 11 through 20 were prepared from aortic samples with calcified type IV atherosclerotic plaques, and RNA samples 21 through 30 were prepared from material removed at carotid endarterectomy. Southern blots of RT-PCR reactions were performed with equal amounts of cDNA, and chemokine expression levels are shown in Figure 3. Approximately equal amounts of β2-microglobulin RT-PCR product were obtained with each cDNA sample. The level of fractalkine mRNA relative to a reference mRNA, β2-microglobulin, varies >70-fold
between different tissue samples (Figure 3). The highest level of fractalkine expression is seen in aortic and carotid tissue samples that contain advanced atherosclerotic plaques (samples 18, 19, and 27). The level of MDC mRNA expression relative to $\beta_2$-microglobulin varies widely between arterial samples (Figure 3), but the highest levels of expression are seen in aortic and carotid arterial samples with complex atherosclerotic lesions. A similar pattern of expression is seen for TARC. A correlation between the level of MDC and TARC expression can be seen in some but not all of the carotid tissue samples (eg, samples 23, 27, 28, and 30). Interestingly, the carotid arterial sample with the lowest level of MDC and TARC expression (sample 25) had the highest level of MCP-1 expression (data not shown).

Fractalkine, MDC, and TARC Expression in Human Atherosclerotic Lesions

We have developed an anti-fractalkine antiserum that recognizes the intracellular domain of the transmembrane fractalkine molecule. Using an antibody that specifically recognizes only the intracellular portion of fractalkine allows us to detect cells that express fractalkine in vivo. We used this antiserum and reagents that specifically detect human MDC and TARC to examine chromosome 16q13 chemokine expression in human atherosclerotic lesions. Figure 4 shows immunohistochemical analysis of serial sections of an atherosclerotic plaque in a carotid artery removed at endarterectomy. Staining with an anti-CD68 antibody shows the presence of large numbers of macrophages within the carotid plaque (Figure 4A). An anti–smooth muscle actin antibody shows that there are very few smooth muscle cells within the macrophage-rich region of the plaque except for those present in arterioles (Figure 4B). The specificity of antibody staining was demonstrated by using an isotype-matched mouse monoclonal antibody (Figure 4C). An affinity-purified antiserum that specifically detects MDC stains a subset of macrophages within the shoulder region of the atherosclerotic plaque stain but does not stain the endothelium of intraplaque microvessels (Figure 4D); no MDC staining was seen in a normal radial arterial sample (data not shown). A very similar pattern of expression is seen with the use of an antiserum that specifically recognizes the intracellular domain of fractalkine (Figure 4E). An antiserum that has been reported to detect the chemokine domain of fractalkine stains macrophages, smooth muscle cells, and endothelium in an adjacent section of the same lesion (Figure 4F). This antibody stains smooth muscle cells and endothelium in normal arterial samples (data not shown). We have shown that this anti-fractalkine reagent cross-reacts with recombinant human CD84, and this most likely accounts for the different staining pattern obtained with the different anti-fractalkine reagents. A similar pattern of chemokine expression was seen in 6 independent carotid endarterectomy samples (see Table).

We extended our immunohistochemical analysis to atherosclerotic lesions in femoral and popliteal arteries obtained after surgical procedures (see Table). Figure I (which can be accessed online at http://atvb.ahajournals.org) shows immu-
**Discussion**

We have analyzed the expression pattern of the linked chemokine genes MDC, fractalkine, and TARC in normal human arteries and in atherosclerotic lesions. All 3 chemokines are expressed in human atherosclerotic plaques by a subset of macrophages within the atherosclerotic lesion, often associated with regions of neovascularization. We have shown that MDC, fractalkine, and TARC expression by primary human macrophages is upregulated by treatment with the Th2-type cytokines IL-4 and IL-13 but not by the IL-10 or the Th1-type cytokine IFN-γ.

**Chromosome 16q13 Chemokine Expression as a Surrogate Marker of Th2-Type Immune Responses?**

We have shown that the genes encoding the CC chemokines MDC and TARC are physically linked to the gene encoding the CX3C chemokine fractalkine on chromosome 16q13. The majority of the CC chemokine genes in the human genome have been mapped to a locus on chromosome 17q11.2. The murine homologues of MDC, fractalkine, and TARC, ABCD-1, ABCD-2, and ABCD-3, respectively, are also physically linked in the murine genome. It is striking that the expression of all 3 chemokine mRNAs in human macrophages is upregulated by treatment with the Th2-type cytokine IL-4 (Figure 1B and 1C). The very similar pattern of MDC, fractalkine, and TARC expression by macrophages within complex atherosclerotic plaques (Figure 4 and online Figure I) suggests coordinate regulation of these linked chemokines in vivo, potentially by Th2-type cytokines. The measurement of chemokine mRNA levels in human arterial tissue samples revealed a wide range of levels of expression (Figure 3), especially for the chemokine MDC, and it is possible that other cytokines and inflammatory stimuli might regulate chromosome 16q13 chemokine expression within the arterial wall.

Recent studies of the cytokine secretion profile of T lymphocytes recovered from human atherosclerotic plaques have suggested that the majority of CD4+ T cells in unstable atherosclerotic plaques secrete IFN-γ and, hence, are more likely to be associated with a Th1-type immune response. Mach et al have reported that the CXC chemokines IP-10, Mig, and I-TAC are expressed in human atherosclerotic lesions. All 3 CXC chemokines are induced by IFN-γ; hence, their expression might be considered a surrogate marker of Th1 cytokine expression in human atherosclerotic lesions. The same group recently reported expression of the CC chemokine eotaxin and its receptor CCR3 in atherosclerotic lesions. The eotaxin receptor CCR3 is preferentially expressed by Th2 T cells, and an increased number of CCR3-expressing T cells are seen in pathologies characterized by Th2-type immune responses, such as atopic dermatitis. It is possible to envisage competing programs of Th1 and Th2 cytokine expression within the microenvironment of the developing atherosclerotic plaque. Th1-type cytokines would lead to macrophages expressing a more inflammatory program of gene expression, whereas a Th2-type immune response might give rise to a macrophage gene expression program more associated with neovascularization and fibrosis. It is interesting that hypercholesterolemia leads to a switch from Th1-type to Th2-type immune responses in apoE-knockout mice.

**Role of MDC, Fractalkine, and TARC in Mononuclear Cell Recruitment and Retention Within Atherosclerotic Lesions**

Soluble fractalkine can recruit monocytes, T lymphocytes, and NK cells via chemotaxis, and immobilized forms of fractalkine have been shown to mediate tight adhesion independent of the expression of other endothelial cell surface receptors. The recent demonstration that immobilized recombinant fractalkine can mediate the tight adhesion of monocytes and T cells under flow conditions makes the...
TABLE 1. Arterial Samples Used in Immunohistochemical Experiments

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age at Op, y</th>
<th>HT</th>
<th>Diabetes</th>
<th>IHD</th>
<th>PVD</th>
<th>CVA/TIA/AF</th>
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<td>Carotid (ulcerated plaque)</td>
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<td>No</td>
<td>?</td>
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</tr>
<tr>
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<td>Leg</td>
<td>No</td>
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<td>M</td>
<td>83</td>
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<td>No</td>
<td>No</td>
<td>Leg</td>
<td>CVA</td>
<td>Femoral artery</td>
</tr>
<tr>
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<td>M</td>
<td>65</td>
<td>No</td>
<td>NIDDM</td>
<td>No</td>
<td>Leg</td>
<td>No</td>
<td>Femoral artery (calcified plaque)</td>
</tr>
<tr>
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<td>72</td>
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<td>No</td>
<td>MI</td>
<td>Leg</td>
<td>No</td>
<td>Femoral artery (calcified plaque)</td>
</tr>
</tbody>
</table>

Op indicates operation; HT, hypertension; IHD, history of ischemic heart disease; PVD, evidence of peripheral vascular disease; CVA, history of cerebrovascular accident; TIA, transient ischemic attack; and AF, atrial fibrillation.

Tissue samples were obtained from patients undergoing surgical procedures. Immunohistochemical analysis of chemokine expression in serial sections of the listed arteries showed macrophage expression of MDC, fractalkine, and TARC expression in atherosclerotic lesions. Representative data are shown in Figure 4 and online Figure I (please see http://atvb.ahajournals.org).

References


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