Raised MCP-4 levels in symptomatic carotid atherosclerosis: an inflammatory link between platelet and monocyte activation

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Aims

Several studies suggest a pro-atherogenic role for the CC chemokine receptor 2 (CCR2), thought to reflect interaction with monocyte chemoattractant protein (MCP)-1. Based on its ability to attract leucocytes into inflamed tissue, we hypothesized a pro-atherogenic role for MCP-4, another CCR2 ligand.

Methods and results

Our main findings were: (i) patients with symptomatic carotid stenosis (n = 29), but not those with asymptomatic plaques (n = 31), had significantly raised plasma levels of MCP-4 compared with healthy controls (n = 20); (ii) in vitro, releasate from activated platelets markedly increased the expression of MCP-4 and CCR2 in THP-1 monocytes, and enhanced the MCP-4-mediated effect on interleukin-8 secretion in these cells, involving the platelet-derived chemokine RANTES; (iii) while MCP-1 had no effect on the release of RANTES and interferon-inducible protein of 10 kDa in tumour necrosis factor α-pre-activated THP-1 monocytes, MCP-4 profoundly enhanced the release of these pro-atherogenic chemokines; and (iv) the data indicate an inflammatory interaction between RANTES and MCP-4, involving CCR2, and mRNA levels of these mediators were markedly up-regulated within symptomatic atherosclerotic carotid plaque (n = 81).

Conclusion

Our findings suggest that the pro-atherogenic effects of CCR2 may not be restricted to interaction with MCP-1, but could also involve activation by MCP-4, being an inflammatory link between platelet and monocyte activation.

Keywords

Atherosclerosis • Inflammation • Platelets • Monocytes

1. Introduction

While the concept that atherosclerosis is an inflammatory disease is no longer controversial, our understanding of the regulation of this inflammatory process as well as the identification and characterization of the different factors are not complete. Several lines of evidence suggest that chemokines are important mediators in this immune-mediated process, leading to progression of atherosclerosis and plaque destabilization. A number of studies have reported raised levels of chemokines in atherosclerosis, both systemically and within the atherosclerotic plaques. Targeted disruption of the genes for monocyte chemoattractant protein-1 (MCP-1)/CCL2, CCR2 (i.e. MCP-1 receptor), CXCR2 (i.e. the interleukin-8 (IL-8)/CXCL8 receptor), CXCR6 (i.e. CXCL16 receptor), and CX3CR1 (i.e. fractalkine/CX3CL1 receptor) significantly decreases atherosclerotic lesion formation in mice prone to develop atherosclerotic-like lesions. Combined inhibition of MCP-1, CX3CR1, and CCR5 in ApoE-deficient mice leads to an additive reduction in atherosclerosis.
indicating that successful therapeutic strategies may need to target multiple chemokines/receptors.\(^9\)

MCP-4/CCL13 signals through the chemokine receptors CCR2, CCR3, and CCR5, all shown to be involved in atherogenesis.\(^2\)

MCP-4 is known as a high efficacy chemoattractant, inducing responses in eosinophils, monocytes, and T cells, and has been implicated in the pathogenesis of various inflammatory disorders such as rheumatoid arthritis and asthma.\(^10\)\(^11\) However, while MCP-1 is regarded as a prototypical pro-atherogenic chemokine, very few studies have examined the role of MCP-4 in atherosclerosis.

Based on its role in inflammation, acting through ‘pro-atherogenic’ chemokine receptors, we hypothesized a role for MCP-4 in atherogenesis and plaque destabilization. In the present study, we investigated this hypothesis by differential experimental approaches including in vivo studies in patients with carotid plaques and in vitro studies in cells with relevance to atherogenesis (i.e. monocytes and platelets).

### 2. Methods

#### 2.1 Patients and controls

For the plasma sample study, 60 consecutive patients at Rikshospitalet, Oslo, Norway, with high-grade internal carotid stenoses (≥70%), treated with carotid endarterectomy (n = 52) or carotid angioplasty with stenting (n = 8), were recruited (Table 1). The patients were classified into three groups according to their plaque symptomatology. Fifteen (25%) patients had suffered from clinical symptoms such as stroke, transient ischemic attack (TIA), or amaurosis fugax ipsilateral to the stenotic internal carotid artery within the past 2 months, 14 (23%) patients had symptoms within 2 to 6 months ago, and 31 (52%) patients were characterized as asymptomatic (symptoms >6 months ago or had never suffered from symptoms as outlined above) (Table 1). Asymptomatic carotid stenosis was detected during clinical examinations of patients with coronary artery disease (CAD), peripheral artery disease, or stroke/TIA >6 months ago. The carotid stenoses were diagnosed and classified by pre-cerebral colour Duplex and CT angiography according to consensus criteria. The plaques were also divided into two groups (i.e. echoluent or echogenic/heterogeneous) depending on plaque echogenicity.\(^12\) Patients with concomitant inflammatory disease (e.g. infection or autoimmune disorder), malignancies, and liver or kidney disease were excluded. For comparisons, blood samples were also collected from 20 sex- and age-matched healthy controls. Platelet-poor plasma (EDTA) were collected from all individuals.\(^12\)

### 2.2 Tissue sampling from carotid plaque

For immunohistochemistry, atherosclerotic carotid plaques were retrieved from patients during carotid endarterectomy at Rikshospitalet, Oslo. Plaques were washed in PBS, mounted in an optimal cutting temperature compound for cryosectioning, snap-frozen on dry ice, and stored at −80°C until immunohistochemical analysis. For the Gene Array analysis, human plaque tissue was obtained from the Biobank of Karolinska Endarterectomies (BIKE) study (Stockholm, Sweden).\(^13\) Samples of atherosclerotic tissue, collected from patients with symptomatic carotid plaques (i.e. symptoms within the past 6 months, n = 81), undergoing carotid endarterectomy, were washed in PBS, and immediately frozen and stored at −80°C until RNA extraction. Control tissue was obtained from iliac arteries of organ donors (n = 10).

### 2.3 Cell culture experiments

The human monocyte cell line THP-1 (American Type Culture Collection, Rockville, MD, USA) were cultured for 4 days in RPMI 1640 (PAA laboratories, Pasching, Austria) supplemented with 2.5% foetal bovine serum (Gibco, Grand Island, NY, USA) in the presence of recombinant human tumour necrosis factor α (rhTNFα, 5 ng/mL, R&D Systems,

### Table 1 Baseline variables in patients according to clinical symptoms and months after symptoms\(^a\) (n = 60)

<table>
<thead>
<tr>
<th></th>
<th>≤2 months (n = 15)</th>
<th>&gt;2 and &lt;6 months (n = 14)</th>
<th>≥6 months or no symptoms (n = 31)</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Age, year</td>
<td>65 (56–83)</td>
<td>68 (50–79)</td>
<td>65 (44–81)</td>
<td>0.542</td>
</tr>
<tr>
<td>Male sex(^b)</td>
<td>11 (73.3)</td>
<td>10 (71.4)</td>
<td>21 (67.7)</td>
<td>0.919</td>
</tr>
<tr>
<td>Degree of stenosis, %</td>
<td>80 (80–95)</td>
<td>80 (70–95)</td>
<td>80 (70–95)</td>
<td>0.659</td>
</tr>
<tr>
<td>Echoluent carotid plaque(^b)</td>
<td>6 (40.0)</td>
<td>3 (21.4)</td>
<td>9 (29)</td>
<td>0.544</td>
</tr>
<tr>
<td>Ischaemia on cerebral MRI(^b) (n = 53)</td>
<td>11 (73.3)</td>
<td>9 (64.3)</td>
<td>21 (67.7)</td>
<td>0.831</td>
</tr>
<tr>
<td>Body mass index, kg/m(^2)</td>
<td>26.7 (20.4–35.5)</td>
<td>25.2 (21.2–28.2)</td>
<td>26.6 (19–33.5)</td>
<td>0.725</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>151 (110–196)</td>
<td>162 (110–193)</td>
<td>150 (110–94)</td>
<td>0.500</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>73 (32–99)</td>
<td>85 (58–101)</td>
<td>78 (49–99)</td>
<td>0.056</td>
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<tr>
<td>Statin treatment(^b)</td>
<td>13 (86.7)</td>
<td>12 (85.7)</td>
<td>28 (90.3)</td>
<td>0.881</td>
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<tr>
<td>Current smoking(^b)</td>
<td>7 (46.7)</td>
<td>8 (57.1)</td>
<td>16 (51.6)</td>
<td>0.789</td>
</tr>
<tr>
<td>Neopterin, nmol/L</td>
<td>8.6 (4.9–16.0)</td>
<td>11.8 (7.6–30.9)</td>
<td>8.6 (5.7–60.3)</td>
<td>0.005</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>3.3 (1.0–27.0)</td>
<td>5.0 (1–21)</td>
<td>5.0 (0.6–28.0)</td>
<td>0.727</td>
</tr>
<tr>
<td>Total leucocyte count, 10(^9)/L</td>
<td>8.3 (4.0–12.1)</td>
<td>7.8 (4.8–10.6)</td>
<td>7.5 (3.8–11.8)</td>
<td>0.520</td>
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<tr>
<td>Cholesterol, mmol/L</td>
<td>4.3 (3.6–3.9)</td>
<td>4.7 (3.7–6.3)</td>
<td>4.2 (2.8–7.5)</td>
<td>0.102</td>
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<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.3 (0.7–1.9)</td>
<td>1.2 (0.8–2.2)</td>
<td>1.3 (0.8–2.5)</td>
<td>0.987</td>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>1.3 (0.6–3.8)</td>
<td>1.9 (0.7–3.7)</td>
<td>1.4 (0.7–3.5)</td>
<td>0.572</td>
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<tr>
<td>LDL (n = 47)</td>
<td>2.6 (2.0–4.0)</td>
<td>2.6 (1.8–4.6)</td>
<td>2.6 (1.5–5.1)</td>
<td>0.342</td>
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<tr>
<td>HbA1c, %</td>
<td>5.7 (5.2–6.9)</td>
<td>5.8 (4.4–7.8)</td>
<td>5.7 (0.9–8.8)</td>
<td>0.995</td>
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<tr>
<td>β-thromboglobulin, IU/mL</td>
<td>50.6 (26.7–142.1)</td>
<td>56.4 (37.7–215.2)</td>
<td>53.0 (15.4–139.9)</td>
<td>0.383</td>
</tr>
<tr>
<td>Platelet count, 10(^9)/L</td>
<td>221 (132–353)</td>
<td>262 (187–441)</td>
<td>264 (168–353)</td>
<td>0.106</td>
</tr>
</tbody>
</table>

\(^a\)Clinical symptoms include stroke, TIA, or amaurosis fugax ipsilateral to the stenotic internal carotid artery. Values are median (range) or numbers (percentages).
Minneapolis, MN, USA) before further incubation with or without different concentrations of rhMCP-4, rhMCP-1, rhIL-1β (5 ng/mL) (all from R&D Systems), different concentrations of rh Regulated on Activation, Normal T cell Expressed and Secreted (RANTES/CCL5, R&D Systems), lipopolysaccharide (LPS) from E. coli 026:B6 (5 ng/mL, Sigma, St Louis, MO, USA), a toll-like receptor (TLR)2 agonist (Pam3Cys, 1 μg/mL, Sigma), isoproterenol (20 μM, Sigma), or platelet releasate (see below). In some experiments, THP-1 monocytes, without pre-incubation with rhTNFα, were stimulated with different concentrations of rhMCP-4 or rhMCP-1, low concentration of rhIL-1β (0.5 ng/mL), or a combination thereof. In a separate set of experiments, peripheral blood mononuclear cells (PBMC) from healthy controls, obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep; Nycomed, Oslo, Norway) gradient centrifugation, were incubated in RPMI 1640 with HEPES (PAA laboratories; 10° cell/mL), with 5% FCS, with or without rhMCP-4 (200 ng/mL, R&D Systems) or platelet releasate (see below). At different time points, cell-free supernatants and cell pellets were harvested and stored at −80°C. In all experiments with controls and un-stimulated cells, the vehicle of the stimulus was always added. The toxicity in cell cultures was examined routinely for lactate dehydrogenase leakage (cytotoxicity detection kit; Roche Applied Science, Indianapolis, IN, USA).

### 2.4 Preparation of platelet releasate

Platelet rich plasma (PRP) was prepared from citrated blood by centrifugation at 270 g for 10 min at 22°C. Preparation of releasates from platelets was performed by adding one-fourth volume of acid-citrate-dextrose to PRP prior to centrifugation at 1500g for 7 min at 22°C as previously described. Briefly, the platelets were then re-suspended in RPMI 1640 media (PAA laboratories) to 10 × 10⁸ platelets/mL before being stimulated with 0.1 U/mL thrombin (Sigma) for 90 min to induce release of platelet components to the media. Hirudin (0.4 U/mL, Sigma) was added to neutralize thrombin. The platelets were then removed by centrifugation at 10 000 g for 5 min at 12°C, and the supernatant, representing platelet releasates from activated platelets, was added to THP-1 cells and PBMC (see earlier). Platelet-free supernatants of un-stimulated PRP that had been incubated for 90 min were also added to THP-1 cells and PBMC, representing platelet releasate from un-stimulated platelets. In some experiments, a LIGHT (TNF superfamily ligand 14 (TNFSF14)) neutralizing, a RANTES neutralizing, a CD40 ligand (CD40L) neutralizing, and in some experiments, a LIGHT [TNF superfamily ligand 14 (TNFSF14)] neutralizing, a RANTES neutralizing, a CD40 ligand (CD40L) neutralizing, and an irrelevant isotype-matched antibody (10 μg/mL, Sigma) were added to neutralize thrombin. The platelets were then removed by centrifugation at 270 000 g for 10 min at 4°C.

### 2.5 Immunohistochemistry

See Methods in the Supplementary methods online.

### 2.6 Real-time quantitative RT–PCR

Total RNA was extracted from THP-1 cells and PBMC using MagNa Pure LC RNA isolation kit III (Roche Applied Science, Oslo, Norway), subjected to DNase I treatment, and stored in RNA storage solution (Ambion, Austin, TX, USA) at −80°C. Primers for CCR2, CCR3, CCR5, and CD68 were designed using the Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA, USA). Primer sequences could be provided by request. Quantification of mRNA was performed using the ABI Prism 7500 (Applied Biosystems). Gene expression of the housekeeping gene β-actin (Applied Biosystems) was used for normalization.

### 2.7 Gene array hybridization and analysis of samples from carotid plaques and control arteries

For the analyses of carotid arteries and control arteries, biotinylated RNA was generated using 10 μg of total RNA. For details, see Methods in the Supplementary methods online.

### 2.8 Miscellaneous

MCP-1, MCP-4, IL-8, RANTES, and interferon-inducible protein of 10 kDa (IP-10/CXCL10) levels were measured by enzyme immunoassays (EIA, R&D Systems). Plasma levels of neopterin and β-thromboglobulin (β-TG) were measured by EIA (IBL Hamburg, Hamburg, Germany and Diagnostica Stago, Asnières, France, respectively). C-reactive protein levels were determined by a high-sensitivity particle enhanced immunoturbidimetric assay on a Modular platform (Roche Diagnostic, Basel, Switzerland). The intra- and inter-assay coefficient of variation was <10% for all assays.

### 2.9 Ethics

The protocols were approved by the Regional Health Authorities of South-Eastern Norway. The investigation confirms with the principles outlined in the Declaration of Helsinki for use of human tissue or subjects. Signed informed consent for participation in the study was obtained from all individuals.

### 2.10 Statistical analysis

For comparison of two groups of individuals, the Mann–Whitney U test was used. For comparison of >2 groups, the nonparametric Kruskal–Wallis test was used. If a significant difference was found, Mann–Whitney U test was used to calculate the difference between each pair of groups. In the in vitro studies, Student’s t-test or Mann–Whitney U test was used depending on the distribution of data. The Chi-square test was used for analysing contingency data. Coefficients of correlation were calculated by the Spearman’s rank test. Multiple logistic regression analysis was performed to investigate the specific influence of covariates. Probability values (two-sided) were considered significant at P < 0.05.

### 3. Results

#### 3.1 Plasma levels of MCP-4 in patients with carotid stenosis and healthy controls

When patients were divided into three groups according to their latest clinical symptoms [i.e. symptoms within the last 2 months (n = 15), symptoms within the last 2–6 months (n = 14), or asymptomatic plaques (n = 31)], plasma levels of MCP-4 significantly above those of matched healthy controls were found in patients with symptomatic, but not in those with asymptomatic carotid stenosis, with particularly high levels in patients with the most recent symptoms (i.e. within 2 months) (Figure 1). In contrast, most of the parameters in Table 1, including the degree of stenosis, lipid parameters, smoking habits, the fraction of patients with echolucent plaques, and plasma levels of C-reactive protein, were not different among the three groups of patients. Plasma levels of MCP-4 were positively correlated with age (r = 0.30, P = 0.02) and β-TG (r = 0.39, P = 0.002), and negatively correlated with platelet counts (r = −0.35, P = 0.006), but in contrast to MCP-4, none of these parameters were related to plaque symptomatology (Table 1). In fact, in addition to MCP-4, neopterin, a reliable marker of monocyte/macrophage activation, was the only other parameter that was significantly different between the three groups of patients (Table 1), and...
multivariate analyses showed that the association of MCP-4 with symptomatic carotid plaques was independent of neopterin ($P < 0.05$). Furthermore, while the highest MCP-4 levels were seen in patients with the most recent symptoms, the highest neopterin levels were seen in those with symptoms between 2 and 6 months ago (Table 1), suggesting that MCP-4 may more accurately reflect plaque instability than neopterin.

### 3.2 The regulation of MCP-4 and CCR2 in THP-1 monocytes

Monocytes and macrophages are key cells in atherogenesis and plaque destabilization. To further elucidate the potential role of MCP-4 in atherosclerosis, we examined the expression of MCP-4 and its receptors in THP-1 monocytes that had been activated by different stimuli with relevance to atherogenesis (i.e. inflammatory mediators, neurohormones, and platelets). TNF$\alpha$ is released from T cells and macrophages during inflammation, playing a major role in the inflammatory interaction between these cells during atherogenesis,$^{16,17}$ and there are several report of enhanced TNF$\alpha$ expression within atherosclerotic carotid plaques.$^{18,19}$ Before activation, the THP-1 cells were therefore exposed to rhTNF$\alpha$ (5 ng/mL) for 96 h, trying to mimic the inflammatory microenvironment within an atherosclerotic lesion. After being exposed to TNF$\alpha$, the cells were still in suspension, and although they expressed CD68 (real-time RT–PCR), the levels were lower than in adherent macrophages ($\sim$75% lower). Hence, the TNF$\alpha$-exposed THP-1 cells could be characterized as activated monocytes, with some degree of macrophage differentiation, potentially resembling migrating monocytes soon after their entrance to the plaque. Releasate from thrombin-activated platelets, but not from un-stimulated platelets, significantly increased MCP-4 levels in cell supernatants with maximum release after 24 h (Figure 2A). Although the magnitude of the MCP-4 response differed between different platelet donors, the ability of releasate from thrombin activated platelets to induce MCP-4 release in THP-1 cells was consistently found in all experiments. Also, the $\beta$-adrenergic receptor agonist isoproterenol (20 $\mu$M), LPS (TLR4 agonist, 5 ng/mL), and particularly the TLR2 agonist Pam$_3$Cys (1 $\mu$g/mL), but not rhIL-1$\beta$ (5 ng/mL), significantly increased MCP-4 levels with maximum release after 48 h (Figure 2B). Moreover, platelet releasate, but not any of the other stimuli, significantly enhanced mRNA levels of CCR2 after culturing for 6 h, with particularly enhancing effects of releasate from thrombin-activated platelets (Figure 2C).
3.3 The platelet-mediated increase in MCP-4/CCR2 expression in THP-1 monocytes involves RANTES

The inflammatory interaction between platelets and TNFα-exposed monocytes/macrophages may be relevant to the in vivo situation within an atherosclerotic plaque, and the significant correlation between plasma levels of MCP-4 and β-TG, a reliable marker of platelet activation, in patients with carotid plaque, further suggests that MCP-4 could be involved in this inflammatory loop. We therefore examined the platelet-mediated increase in MCP-4/CCR2 expression in THP-1 monocytes in more detail. Releasate of thrombin-activated platelets in itself did not contain any detectable amounts of MCP-4, and the influence of microparticles, released from platelets during activation, seemed to be negligible as ultracentrifugation of platelet releasate did not influence its effect on MCP-4/CCR2 expression (data not shown). Thrombin is a well-known activator of monocytes, but before releasate from thrombin-activated platelets were exposed to THP-1 cells, hirudin was added to the suspension to neutralize any thrombin effect. Indeed, thrombin in combination with hirudin to atherogenesis in THP-1 monocytes that had been pre-activated by rhTNFα, 5 ng/mL that were co-stimulated with releasate from thrombin-activated platelets, suggesting that the platelet-mediated up-regulation of CCR2 enhances its functional responsiveness (Supplementary material online, Figure S1A). Again, this enhancing effect of platelet releasate on the MCP-4-inducing effect of IL-8 was significantly attenuated by neutralizing antibody against RANTES, with no attenuating effect of an irrelevant isotype-matched control antibody (Supplementary material online, Figure S1B).

3.4 Effects of MCP-4 on chemokine release in THP-1 monocytes

Next, we examined the release of various chemokines with relevance to atherosclerosis in THP-1 monocytes that had been pre-activated by rhTNFα (5 ng/mL) for 96 h before MCP-4 stimulation. As shown in Figure 4A and 8, rhMCP-4 markedly enhanced the release of RANTES and IP-10 in THP-1 monocytes after culturing for 24 h. In order to see if the stimulatory effect of MCP-4 in THP-1 monocytes was restricted to pre-activation with TNFα for 96 h, THP-1 cells were stimulated with MCP-4, without any pre-activation with TNFα, with and without co-stimulation with low concentration of IL-1β (0.5 ng/mL). Our findings showed (Figure 4C and D): (i) MCP-4 induced the release of IL-8 without any pre-activation or co-stimuli. (ii) Co-stimulation with IL-1β boosted the MCP-4-mediated release of IL-8, and MCP-4 boosted the IL-1β-mediated effect on IP-10. Importantly, IL-1β, even in high concentrations, had no effect on MCP-4 levels in these cells (Figure 2B), suggesting that the boosting effect of this cytokine does not merely reflect increased MCP-4 release.

3.5 Platelet releasate enhances the MCP-4-mediated effect on IL-8 secretion in THP-1 monocytes

While rhMCP-4 had no effects on its own, it boosted the IL-8 response in THP-1 monocytes (pre-activated by rhTNFα, 5 ng/mL) that were co-stimulated with releasate from thrombin-activated platelets, suggesting that the platelet-mediated up-regulation of CCR2 enhances its functional responsiveness (Supplementary material online, Figure S1A). Again, this enhancing effect of platelet releasate on the MCP-4-inducing effect of IL-8 was significantly attenuated by neutralizing antibody against RANTES, with no attenuating effect of an irrelevant isotype-matched control antibody (Supplementary material online, Figure S1B).

3.6 MCP-4 and MCP-1 exert different responses in THP-1 monocytes

MCP-1 is a prototypical pro-atherogenic chemokine, also signalling through CCR2. Indeed, plasma levels of MCP-1 showed the same pattern as for MCP-4, with increased levels in patients with carotid stenosis with the most recent symptoms (i.e. within 2 months) when compared with asymptomatic patients, although the difference was not so pronounced as for MCP-4 (see Supplementary material online, Figure S2). Moreover, in contrast to MCP-4 (Figure 1), MCP-1 levels in patients with carotid plaques were not different from plasma levels in healthy controls (see Supplementary material online, Figure S2). Also, releasate from thrombin-activated platelets significantly enhanced the release of MCP-1 in THP-1 cells (38 ± 3.7 vs. 81 ± 2.3 pg/mL, un-stimulated and thrombin stimulated, respectively; P < 0.05, n = 4). However, while MCP-1 is known to increase CCR2 expression, neutralizing antibodies against MCP-1 did not influence the enhancing effect of platelet releasate on CCR2 expression in THP-1 cells (Supplementary material online, Figure S3A). Additionally, in contrast to MCP-4, rhMCP-1 had no effect on RANTES and IP-10 release in TNFα pre-activated THP-1 cells (Supplementary material online, Figure S3B–C). In fact, low dose of MCP-1 attenuated the release of RANTES in these cells. Furthermore, in contrast to MCP-1 (Figure 4C), MCP-1 had no effect on RANTES release in un-stimulated THP-1 monocytes (Supplementary material online, Figure S3D). However, like MCP-4, MCP-1 enhanced the release of RANTES and IP-10 in THP-1 monocytes when co-stimulated with IL-1β (0.5 ng/mL) (Supplementary material online, Figure S3D–E), suggesting that these chemokines may promote distinct but also overlapping effects in THP-1 cells.

3.7 Interaction between MCP-4 and RANTES in platelet-exposed PBMC

To verify that our findings on MCP-4 were not restricted to THP-1 monocytes, PBMC from six healthy donors, without any pre-activation by TNFα, were exposed to releasate from thrombin-activated platelets. Like in THP-1 cells, releasate from activated platelets induced the release of MCP-4 in PBMC after culturing for 24 h, and again, this enhancing effect on MCP-4 release was significantly attenuated by neutralizing antibodies against RANTES (Figure 5A). Moreover, the RANTES-inducing effect of MCP-4 (200 ng/mL) was also seen in PBMC after culturing for 24 h (Figure 5B). These findings in freshly isolated PBMC, representing
interacting leucocyte subsets, underscore the in vivo relevance of our findings.

### 3.8 Enhanced expression of MCP-4, RANTES, and CCR2 within atherosclerotic carotid plaques

Our findings so far may suggest an inflammatory interaction between RANTES and MCP-4, involving CCR2-related mechanisms. To further elucidate the in vivo relevance of these findings, we analysed the mRNA levels of MCP-4, RANTES, and CCR2 in atherosclerotic tissue, collected from patients with symptomatic carotid plaques, undergoing carotid endarterectomy \( (n = 81) \), and in control tissue, obtained from iliac arteries of organ donors \( (n = 10) \), by means of Affymetrix Gene Array analysis. As shown in see Supplementary material online, Figure S4, the mRNA levels of all these mediators were markedly up-regulated within the atherosclerotic carotid plaque, with no relation to the degree of stenosis (data not shown). Moreover, immunohistochemical examination of carotid plaques from four patients with symptomatic \( (<2 \text{ months}) \) and four patients with asymptomatic disease showed immunostaining against MCP-4, CCR2, and RANTES in symptomatic plaques that were localized to the lipid-rich core of the lesion, with strong immunostaining against calprotectin-positive macrophages (Figure 6). MCP-4, CCR2, and RANTES immunostaining was also seen in lipid-rich calprotectin-positive regions in lesions from patients with asymptomatic disease. However, the atherosclerotic plaques were less advanced in these patients and the region with MCP-4-, CCR2-, and RANTES-positive immunostaining was smaller (data not shown).

### 4. Discussion

Several studies have suggested a pro-atherogenic role for CCR2, thought to reflect interaction with the pro-atherogenic chemokine MCP-1.\(^2\) The present study suggests that the pro-atherogenic role of CCR2 also involves interaction with MCP-4. Thus, we show (i) markedly elevated levels of MCP-4 in patients with symptomatic carotid atherosclerosis both in plasma and within the lesion, primarily located to monocytes/macrophages. Moreover, (ii) while MCP-1 had no effect on the release of RANTES and IP-10 in TNF-\(\alpha\)-pre-activated monocytes, MCP-4 markedly enhanced the release of these pro-atherogenic chemokines. Our findings therefore indicate that the pro-atherogenic effects of CCR2 may not be restricted to interaction with MCP-1, but could also involve activation by MCP-4. In fact, based on our in vitro findings showing differences in the effect of these two chemokines in THP-1 cells, it is tempting to hypothesize that while both these CCR2 ligands are involved in atherogenesis,
they could, at least partly, represent different inflammatory pathways in this process. However, our data also show similarities between these two CCR2 ligands in relation to effects on chemokine release in THP-1 cells, at least partly depending on the nature of the co-stimuli (IL-1β vs. TNFα), illustrating that these chemokines may promote distinct but also overlapping effects during atherogenesis.

While there are numerous studies on raised circulating MCP-1 levels in atherosclerotic disorders, few studies have examined MCP-4 in these disorders. Recently, Ardigo et al.20 showed that the combination of serum levels of multiple chemokines, including MCP-4, could indentify subjects with clinically significant CAD. In the current study, we show a significant relation between plasma levels of MCP-4 and plaque symptomatology in patients with carotid atherosclerosis. Thus, while there were no differences in plasma levels of MCP-4 between healthy controls and patients with asymptomatic carotid stenosis, patients with the most recent symptoms had markedly increased MCP-4 levels. Previously, Berkhout et al.21 reported that MCP-4 was strongly associated with macrophages in the two carotid endarterectomy specimens they examined. Here, we extend these findings by showing significantly increased MCP-4 in atherosclerosis.

Figure 4 Inflammatory effects of MCP-4 in THP-1 monocytes. The effect of different concentrations of MCP-4 on the release of RANTES (A and C) and IP-10 (B and D) in THP-1 monocytes after culturing for 24 h. In (A) and (B), THP-1 cells were pre-activated with TNFα (5 ng/mL) for 96 h prior to MCP-4 stimulation, without any co-stimulation with IL-1β. In (C) and (D), the THP-1 cells (without any pre-activation) were cultured with and without IL-1β (0.5 ng/mL). RANTES and IP-10 levels were measured by EIA. Data are mean ± SEM [n = 4 (A and B) and n = 5 (C and D)]. *P < 0.05 vs. controls (Ctrl). #P < 0.05 vs. 500 ng/mL MCP-4 without IL-1β; £P < 0.05 vs. IL-1β alone.

Figure 5 Interaction between MCP-4 and RANTES in platelet-exposed PBMC. (A) shows the effects of platelet releaseate from un-stimulated (uPRL) and thrombin-activated (sPRL) platelets on the release of MCP-4 in PBMC after culturing for 24 h with or without neutralizing antibodies (Ab) against RANTES and isotype control antibodies (10 μg/mL for both). Neutralizing antibodies against CD40L and LIGHT had no effects on the sPRL-induced MCP-4 release (data not shown). (B) shows the effect of MCP-4 (200 ng/mL) on RANTES release in PBMC after culturing for 24 h. Data are mean ± SEM (n = 6). *P < 0.05 vs. control (Ctrl). **P < 0.01 vs. Ctrl and neutralizing Ab against RANTES.
increased MCP-4 expression in symptomatic carotid lesion, examining plaques from 81 patients. Our immunohistochemistry data further support a link between MCP-4 and macrophages within the lesion.

Our in vitro findings suggest a relationship between enhanced MCP-4 levels in monocytes/macrophages and stimuli with relevance to atherosclerosis such as TLR agonists, β-adrenergic receptor activation, and platelets. Also, we have previously shown raised IL-1 levels in atherosclerotic disorders,22 and while this cytokine had no effect on MCP-4 expression in THP-1 monocytes on its own, even low concentrations of IL-1β enhanced the MCP-4-mediated effect on chemokine release in these cells. Our findings therefore suggest that MCP-4 is part of an inflammatory network involving platelets, cytokines, microbial antigens, and neurohormones, contributing to vascular inflammation within the atherosclerotic lesion.

Upon activation, platelets release the contents of their secretory granules, and this platelet releasate comprises a multitude of inflammatory and vasoactive substances, which can attract atherogenic leucocytes from the circulation and activate endothelial cells and monocytes/macrophages within the atherosclerotic lesion.23 Here, we show that releasate from activated platelets significantly enhances MCP-4 and CCR2 expression in THP-1 monocytes. Moreover, platelet releasate significantly boosted the MCP-4-inducing effect of IL-8 in these cells, suggesting that the platelet-mediated up-regulation of CCR2 is biologically active. While several platelet-derived mediators could be involved in these enhancing effects on MCP-4, CCR2, and their interactions, our findings suggest that platelet-derived RANTES is of particular importance. By the ability of MCP-4 to induce RANTES release in monocytes, the inflammatory interaction between RANTES and MCP-4 could also be operating within...
monocytes/macrophages, leading to increased MCP-4 and RANTES-mediated responses. Of interest, all these mediators (i.e. MCP-4, RANTES, and CCR2) were significantly up-regulated in atherosclerotic plaques from patients with symptomatic carotid stenosis, suggesting that this inflammatory chemokine loop could be operating within the atherosclerotic lesion. RANTES has been shown to be a specific marker of refractory unstable angina pectoris, and is transiently raised during severe ischaemic symptoms. Platelet-derived RANTES triggers monocyte arrest on inflamed endothelium, and recently, inhibition of platelet-derived RANTES was found to attenuate atherosclerosis in hyperlipidaemic mice. Based on the findings in the current study, it is tempting to hypothesize that the pro-atherogenic effects of RANTES also could involve inflammatory interactions with MCP-4/CCR2.

The present study has some limitations such as low numbers of control samples in the clinical part of the study, lack of more accurate evaluation of atherosclerotic inflammatory burden within the carotid plaques (e.g., by positron emission tomography), and lack of data form PBMC in the patient group. However, based on the high expression of MCP-4 within the atherosclerotic plaques, together with its high efficacy as a chemoattractant for monocytes and T cells, we speculate that MCP-4 is involved in the recruitment of monocytes and T cells to atherosclerotic lesions. The ability of MCP-4 to enhance the release of CC (i.e., RANTES) and CXC (i.e., IP-10 and IL-8) chemokines, as demonstrated in the current study, could further contribute to its ability to recruit and activate leucocytes into the atherosclerotic lesion. These inflammatory loops within the chemokine network, involving interactions between platelets and monocytes/macrophages, further support the idea that blocking chemokine ligation could be a therapeutic approach to atherosclerotic disorders. Our findings in the present study show that MCP-4 could be an interesting target in such an approach.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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