Increased Proinflammatory Cytokines in Patients With Chronic Stable Angina and Their Reduction By Aspirin

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Background—Proinflammatory cytokines released by injured endothelium facilitate interaction of endothelial cells with circulating leukocytes and thus may contribute to development and progression of atherosclerosis. We investigated whether cytokines and C-reactive protein (CRP) are indicative of myocardial ischemia or of diseased vessels and whether they are influenced by aspirin treatment in patients with chronic stable angina.

Methods and Results—Plasma macrophage colony stimulating factor (MCSF), IL-1b, IL-6, and CRP were measured in 60 stable patients after 48-hour Holter monitoring and in 24 matched controls. All patients had angiographic documentation of disease and positive exercise ECGs. Patients with ischemia on Holter monitoring (n=40) received aspirin or placebo in a 6-week, randomized, double blind, crossover trial. Blood sampling was repeated at the end of each treatment phase (3 weeks). Compared to controls, patients had more than twice median MCSF (800 versus 372 pg/mL), IL-6 (3.9 versus 1.7 pg/mL), and CRP (1.25 versus 0.23 mg/L) levels (P<0.01 for all comparisons). MCSF was related to ischemia on Holter monitoring (P<0.01), to low ischemic threshold during exercise (P<0.01), and together with IL-1b to number of diseased vessels (P<0.05). MCSF, IL-6, and CRP were all reduced after 6 weeks of aspirin treatment (P<0.05).

Conclusions—These findings suggest that cytokines are associated with both ischemia and anatomic extent of disease in patients with stable angina. Reduced cytokine and CRP levels by aspirin may explain part of aspirin’s therapeutic action.

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Key Words: interleukins • atherosclerosis • coronary disease • ischemia • aspirin

Macrophage colony stimulating factor (MCSF) and IL-1b released by injured endothelium promote the interaction of endothelial cells with circulating leukocytes and may thus contribute to the development and progression of atherosclerosis. MCSF induces the synthesis by endothelial cells of monocyte chemotactic protein 1, which enhances the migration of monocytes into the subendothelial layer. MCSF additionally increases cholesterol uptake by macrophages and delays their apoptosis, resulting in foam cell formation, the hallmark of atherogenesis. MCSF, acting in synergy with IL-1b, induces the activation and proliferation of monocytes/macrophages. These 2 cytokines determine a further release of cytokines from vascular cells, including IL-6 which may be involved in smooth muscle cell proliferation. One of the mechanisms involved in vascular cell activation and proliferation induced by MCSF is mediated by cyclooxygenase activity. The plasma levels of MCSF, IL-6, and C-reactive protein (CRP) have been found elevated in patients with unstable angina and acute myocardial infarction but have not been carefully investigated in patients with chronic stable angina. We hypothesized that MCSF, IL-1b, and IL-6 plasma levels in patients with chronic stable coronary artery disease might be associated with the anatomic extent of disease and that aspirin administration might reduce cytokine plasma levels.

Methods

Study Population

Sixty consecutive patients (53 men, 7 women, mean age 55±5 years, range 38 to 67 years) with clinically stable angina were enrolled. The inclusion criteria were effort angina of at least 1 year’s duration; exercise-induced ischemia; presence of luminal diameter stenosis >50% of 1 or more epicardial coronary arteries at angiography, performed within 1 year of enrolment; and informed consent. Exclusion criteria included ECG evidence of left ventricular hypertrophy or left bundle branch block; acute coronary events, coronary angioplasty, or surgery within the previous 6 months; cerebral vascular disease; peripheral vascular disease; diabetes mellitus; previous coronary artery bypass graft surgery; impaired renal or liver function; bleeding tendencies; allergy to aspirin; malignant or known inflammatory diseases; and age >80 years. On inclusion into the study, antiplatelet drugs were withdrawn for 2 weeks. Holter
TABLE 1. Study Population Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Patients With CSA</th>
<th>Normal Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>55±5 (38-67)</td>
<td>48±13 (32-68)</td>
<td>NS</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>53/7 (87%)</td>
<td>17/7 (71%)</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.9±0.99 (4.8-8.1)</td>
<td>5±0.5 (2-6)</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.8±1.2 (0.78-5.73)</td>
<td>1.2±0.5 (0.8-2)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension, Yes/No</td>
<td>21/39 (35%)</td>
<td>8/16 (33%)</td>
<td>NS</td>
</tr>
<tr>
<td>Family history of CAD, Yes/No</td>
<td>30/30 (50%)</td>
<td>11/13 (46%)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking, Yes/No</td>
<td>34/26 (56%)</td>
<td>13/11 (54%)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of diseased coronary arteries</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11 (18%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>24 (40%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>25 (42%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous MI, Yes/No</td>
<td>31/29</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Medication*</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Medical treatment at inclusion.
†Patients only on one of the 3 classes of antianginal drugs.
‡Patients only on sublingual GTN at inclusion.

CSA indicates chronic stable angina; GTN, glyceryl trinitrate; and CAD, coronary artery disease.

monitoring and blood sampling were then performed. Patients with signs of ischemia on Holter monitoring entered a randomized trial to test the effects of aspirin on cytokine levels.

Twenty-four clinically healthy subjects (17 men, 7 women, mean age 52±13 years, range 32 to 68 years) served as a control group. They had normal baseline ECG, echocardiogram and treadmill test, no evidence of active infection, and were taking no medications. Patients and controls were matched for coronary artery disease risk factors (Table 1) to secure a similar state of oxidative stress. The study protocol was approved by the Hammersmith Hospital’s Ethics Committee.

Exercise Testing
Treadmill exercise testing (Marquette Case 15) was performed according to the Bruce or modified Bruce protocol within 3 months before enrollment (15 to 90 days). Antianginal therapy was withheld for 48 hours before the test. The number of metabolic equivalents (Mets) achieved at ST-segment depression >0.1 mV, 60 ms after the J point, was used to indicate the ischemic threshold.

Holter Monitoring
Patients underwent a 48-hour Holter monitoring (Marquette 8000 laser system) to assess the presence of ischemia during daily activities. The Holter recording was performed no later than 3 months after the exercise test. Antianginal therapy was again withheld for 48 hours before and during monitoring, although sublingual nitrates were allowed if chest pain persisted beyond 3 minutes. Only 7 of 60 patients used sublingual nitrates. Antiplatelet drugs were not administered during the preceding 2 weeks. ST-segment depression >0.1 mV, occurring 60 ms after the J point and lasting at least 1 minute, was considered indicative of ischemia.25 Forty of the 60 enrolled patients (67%) showed signs of ischemia during Holter monitoring.

Blood Sampling
Baseline morning blood samples were taken from the 60 patients, at the end of Holter monitoring, and from controls. The samples were drawn into plastic tubes containing 1:9 volumes of 0.103 mol/L trisodium citrate and centrifuged at 2000g for 15 minutes at 40°C. Aliquots of plasma were stored at -70°C until subsequent analysis.

Randomization to Aspirin or Placebo
The 40 patients with signs of ischemia during Holter monitoring were randomized in a crossover, double blind trial to receive either oral aspirin (300 mg/d) or placebo. Patients without ischemia during the Holter recording were excluded to achieve greater homogeneity of the study population. Aspirin and placebo were provided by the hospital’s pharmacy as identical capsules. Each treatment phase lasted 3 weeks, to avoid any carry over effects of aspirin. Thus, 20 patients received aspirin, and the remaining 20 placebo for 3 weeks; all 40 patients were then crossed over to the alternate treatment for another 3 weeks.

Blood sampling was repeated at the end of each 3-week phase. Antianginal medication, with the exception of sublingual nitrates, was withheld for 4 days before blood sampling. Patients and physicians involved in the trial were blinded to the type of medication (aspirin or placebo) assigned during each phase; the code was broken only after analysis of the data had been completed.

Laboratory Assays
The laboratory measurements were performed by personnel unaware of the clinical data. Plasma MCSF concentrations were measured using a commercial enzyme-linked immunoassay (human MCSF, Quantikine R&D system, Minneapolis, Minn). The sensitivity of the assay is 20 pg/mL. IL-6 and IL-1b were measured by high sensitivity immunoassays (human IL-6 and IL-1b Quantikine [high sensitivity] R&D systems) which detect values as low as 0.094 pg/mL and 100 fg/mL, respectively. CRP was measured using particle-enhanced immunonephelometry (N Latex CRP mono, Behring Diagnostics). This assay detects values as low as 0.175 mg/L. The intra-assay coefficient of variation was <5% for all tests. Cytokine levels were within the assays’ detection limit in all patients and controls. CRP levels were below the detection limit in 6 patients (10%) and 8 controls (33%). In such cases, values were obtained by extrapolation of the assay’s standard curve using the equation: y = 141.956+5070x.

Statistical Analysis
Biochemical data are expressed as medians and interquartiles. Differences within and between groups were analyzed by Wilcoxon signed rank test, Mann Whitney U test or ANOVA (Kruskal-Wallis and Friedman test). Spearman’s rank correlation test was used to assess relations between variables. Multiple relations were tested by stepwise regression analysis. Categorical variables were compared by contingency c2 test. P<0.05 (2-tailed) was considered statistically significant.

Results
Clinical Characteristics
Patients and controls did not differ in age, sex, and risk factor distribution (Table 1). The right coronary artery was involved in 43 patients, the left anterior descending artery in 48, and
the left circumflex in 43 cases (Table 1). All patients had an ejection fraction >55% at left ventriculography.

Cytokines in Patients and Controls

Patients had more than twice the plasma concentrations of MCSF, IL-6, and CRP compared with controls (Table 2). ANOVA showed that MCSF and IL-1b levels increased with the number of diseased vessels (Figure 1). Thus, patients with 3-vessel disease had 2- to 3-fold higher MCSF levels than patients with 2- and single-vessel disease, whereas patients with 2-vessel disease had higher levels than patients with single-vessel disease ($P<0.05$).

Similarly, patients with 3-vessel disease had higher IL-1b levels than patients with single- and 2-vessel disease. Only patients with 3-vessel disease had higher IL-1b plasma levels compared with controls ($P<0.05$). MCSF and IL-1b concentrations in patients with single-vessel disease did not differ from controls. Furthermore, MCSF, IL-1b, and IL-6 levels did not differ between patients with (n = 31) or without previous myocardial infarction.

Relation Between Cytokines and CRP

IL-6 and MCSF concentrations were independently related to CRP ($r=0.58$, $P<0.01$) and $r=0.35$, $P<0.05$, respectively) in patients with chronic stable angina. In controls, however, only IL-6 showed a significant relation to CRP levels ($r=0.55$, $P<0.05$). Additionally, MCSF values were related to IL-1b ($r=0.47$, $P<0.01$). There was no relation between IL-6 and MCSF or IL-6 and IL-1b levels in patients or controls (Figure 2).

Cytokines and Ischemia

On Exercise

Predictably, the number of Mets achieved at the onset of ischemia on effort decreased with the number of diseased vessels ($P<0.001$). MCSF levels showed an inverse relation to the number of Mets during ischemia ($r=-0.50$, $P<0.001$). Median MCSF levels were 1100 pg/mL (range, 852 to 1390) in the 37 patients with ischemia <5 Mets versus 320 pg/mL (range, 308 to 686) in the 23 patients with ischemia >5 Mets ($P<0.001$) (5 mets = 2nd stage of Bruce protocol). The 2 subgroups did not differ in age, sex, or risk factor distribution (Figure 3).

Table 2. Proinflammatory Cytokines and CRP in Patients With CSA vs Normal Controls

<table>
<thead>
<tr>
<th>Patients With CSA (n=60)</th>
<th>Normal Controls (n=24)</th>
<th>$P$</th>
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</thead>
<tbody>
<tr>
<td>MCSF, pg/mL</td>
<td>800 (315–1415)</td>
<td>372 (265–770)</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>3.9 (2.7–5.4)</td>
<td>1.7 (1.3–2.5)</td>
</tr>
<tr>
<td>CRP, mg/mL</td>
<td>1.29 (0.5–3.09)</td>
<td>0.23 (0.17–1.4)</td>
</tr>
<tr>
<td>IL-1b, pg/mL</td>
<td>0.24 (0.19–0.5)</td>
<td>0.2 (0.17–0.47)</td>
</tr>
</tbody>
</table>

Values are expressed as median and 25th and 75th percentile.

Figure 1. a, Comparison of IL-1b plasma levels between patients (n = 60) with single- (I), two- (II), and three- (III) vessel disease and controls (n = 24). b, Comparison of MCSF plasma levels between patients with single- (I), two- (II), and three- (III) vessel disease and controls. Values are expressed as median and 25th and 75th percentiles by the box, largest and smallest nonoutlier values by the lines at the ends of the box, and outlier values by the circles.
During Holter Monitoring

The 40 patients with ischemia on Holter had a higher prevalence of multivessel disease compared with the 20 patients without ischemia. Median MCSF levels were 1100 pg/mL (range, 860 to 1329) in the 40 patients with ischemia versus 330 pg/mL (range, 300 to 926) in the 20 patients without (P < 0.01). Patients with and without ischemia during Holter did not differ in age, sex, risk factor distribution or in mean and maximum heart rate during Holter monitoring. The median number and cumulative duration of ischemic episodes was 8 (range, 6 to 11) and 33 minutes (range, 28 to 65), respectively. No significant relation was detected between cytokine plasma levels and number or duration of ischemic episodes.

Aspirin and Cytokine Levels

Although with substantial overlap, there was a significant reduction of MCSF, IL-6, and CRP after 6 weeks of aspirin administration compared with placebo (Table 3). With aspirin, the levels of IL-6, CRP, and MCSF were reduced by 37% (range, 31% to 53%), 29% (range, 9% to 60%) and 17% (range, 11% to 23%), respectively, compared with placebo (P < 0.05 for all comparisons). Cytokine and CRP levels measured at the end of placebo phase did not differ from the baseline values measured before randomization. IL-1b plasma levels did not differ during the placebo and aspirin phases.

Discussion

This is the first prospective study demonstrating the presence of high MCSF, IL-1b, and IL-6 plasma levels in patients with chronic stable angina. There was also a graded relation between cytokine levels and extent and severity of coronary artery disease, particularly with MCSF. Additionally, we found that after a 6-week period of aspirin administration, there was a reduction of cytokine and CRP plasma levels at a dose used in daily clinical practice (300 mg).

Cytokines and Atherosclerosis

Atherogenetic risk factors, such as oxidized low density lipoprotein, 1,2 tobacco glucoprotein, 26 chlamydial or viral infections, 3–6 and several cytokines 3,5,6,27 may all induce the endothelium to produce MCSF. MCSF stimulates the production of IL-1b3,28 and of further MCSF production 3 by the local endothelium, resident macrophages, and by newly-recruited monocytes from peripheral blood. Additionally, MCSF and IL-1b upregulate the expression of leukocyte adhesion molecules on the endothelial surface and of specific integrins on monocytes, leading to enhanced adhesion of monocytes to the endothelium.5–7

Both, MCSF and IL-1b induce macrophage activation and proliferation which promote further release of cytokines.5,15,17 Cytokine-activated monocyte/macrophages produce increased amounts of IL-6 14,15 which may enter the systemic circulation and lead to increased production of CRP by hepatocytes. 29–31 The above sequence of cytokine production derived from animal studies may explain, at least in part, the presence of increased MCSF, IL-1b, IL-6, and CRP levels in patients with known or suspected coronary artery disease. In the present study, both MCSF and IL-1b plasma levels were associated with the extent of coronary artery disease at angiography. As a result, MCSF plasma levels in patients with 3-vessel disease were manyfold higher than in patients with single- or 2-vessel disease. MCSF and, to a lesser extent, IL-1b, enhance cholesterol uptake from human macrophages by upregulation of their oxidized LDL receptors, resulting in foam-cell formation 9–11 and plaque growth. Indeed, mice genetically deficient in MCSF show decreased progression of atherosclerosis. 32 Additionally, the messenger RNA and protein of MCSF and IL-1b have been isolated in human atherosclerotic lesions. 3,4,8,9 Thus, the relation of MCSF and IL-1b to the anatomic

Table 3. Effects of Aspirin on Proinflammatory Cytokines and CRP Plasma Levels in Patients With CSA

<table>
<thead>
<tr>
<th></th>
<th>Placebo (40)</th>
<th>ASA (40)</th>
<th>P</th>
<th>Controls (24)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCSF (pg/mL)</td>
<td>991 (459–1476)</td>
<td>843 (501–1357)</td>
<td>&lt;0.05</td>
<td>372 (265–770)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>3.5 (3.2–4.6)</td>
<td>2.9 (2.5–3.4)</td>
<td>&lt;0.05</td>
<td>1.7 (1.3–2.5)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>1.4 (0.54–4.05)</td>
<td>1 (0.5–3.1)</td>
<td>&lt;0.05</td>
<td>0.23 (0.17–1.4)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

n = 40 patients with ischemia at Holter randomized to aspirin (20) or placebo (20) and then crossed over. ASA indicates aspirin (300 mg).

Values are expressed as median and 25th and 75th percentile.
extent of disease we found in this study may be suggestive of an important role of growth factors and inflammatory mediators in the progression of atherosclerosis in humans, confirming previous experimental findings.2–6,9–11,32

We found that MCSF levels were related to IL-1b and CRP levels in patients but not in controls. This suggests a relation between potentially atherogenic cytokines and acute phase proteins in the setting of atherosclerosis. IL-6 concentrations, however, were related to CRP levels in both patients and controls, as previously observed.29–31

Cytokines and Myocardial Ischemia

Patients with lower ischemic threshold, as assessed by exercise testing and Holter monitoring, had markedly increased MCSF levels compared with patients with a higher ischemic threshold. MCSF stimulates the endothelium and monocyte/macrophages to release several vasoactive substances,2–6,13,17 and it is involved in the production of procoagulant cytokines such as IL-1b and IL-64,5,37 at sites of injured endothelium. Therefore, MCSF, by increased coronary tone,38,39 impaired vasodilatation,19 and formation of microthrombi40,41 may reduce coronary flow and thus initiate, facilitate, or prolong ischemic episodes.42,43 Alternatively, ischemic episodes per se may induce an increase in circulating MCSF, as has been demonstrated for IL-6.44

Effects of Aspirin

We investigated the effects of a 300 mg daily dose of aspirin on cytokines and CRP, in a randomized, double-blind, placebo-controlled, crossover trial. Compared with placebo, aspirin was associated with a significant and concomitant reduction of IL-6, CRP, and MCSF. The induction of macrophage/monocyte activation and proliferation by MCSF and IL-1b involves cyclooxygenase activity.3–18,19 Activated and proliferating vascular cells produce further MCSF and IL-6,14,17 which may enter into the bloodstream. Aspirin may prevent cyclooxygenase-mediated cell activation and proliferation and reduce the release of these cytokines in blood by acetylation of cyclooxygenase.45 Aspirin administration in patients with chronic stable angina has been associated with increased plasma levels of the antiproliferative cytokine, transforming growth factor-β (TGF-β).46 Interestingly, an inverse relation between TGF-β and MCSF blood levels has been found.22 Thus, an increase in TGF-β levels by aspirin may downregulate MCSF and IL-6 production by vascular cells. Alternatively, aspirin may reduce MCSF and IL-6 through mechanisms related to platelet inhibition. The reduction of CRP by aspirin is likely to be secondary to the reduction in IL-6, because IL-6 is known to stimulate the synthesis of acute phase proteins by the liver.29–31

The modest reduction in cytokine levels after 300 mg of aspirin administration may result from the fact that aspirin exhibits its greatest antiinflammatory action at doses as high as 2 g. In support of this, Bhaghat et al17 found that aspirin inhibited the adverse effects of cytokines on endothelium-dependent dilatation in human veins only at a dose of 1 g and not at 75 mg. Several considerations indicate that the reductions in MCSF, IL-6, and CRP levels by aspirin are not fortuitous findings: (1) because of the strict study design (randomized, double-blind, crossover, placebo-controlled); (2) there was a concomitant reduction in MCSF, IL-6, and CRP; (3) there was a good reproducibility of measurements at baseline and during placebo; and (4) there is a reasonable mechanism behind it.

Study Limitations

The following limitations should be acknowledged. The study was not designed to establish whether raised cytokine levels might be a cause or a consequence of atherosclerosis and/or ischemia. The control group did not undergo coronary angiography and thus subclinical coronary artery disease cannot be excluded. Cytokines were measured in peripheral blood. This may have reduced the sensitivity of the measurements and does not allow conclusions on the release of cytokines in the coronary circulation. Sublingual nitrates are nitric oxide donors and may have affected the plasma levels of cytokines in the 7 patients who made use of them. However, there is no clear evidence that nitrates could affect cytokine levels. Conversely, nitric oxide may be a mediator of IL-6’s actions.6

Conclusions

This is the first study to demonstrate the presence of higher circulating levels of the proinflammatory/atherogenic cytokines MCSF, IL-6, and IL-1b in patients with chronic stable angina compared with healthy controls. MCSF and IL-1b were related to anatomic severity of disease and MCSF with low ischemic threshold. This suggests a graded relation between these cytokines and severity of coronary artery disease. In a randomized placebo-controlled trial, aspirin reduced the circulating levels of MCSF, IL-6, and CRP. This may explain part of the drug’s therapeutic action in patients with ischemic heart disease.

Acknowledgments

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References


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