Folic Acid Treatment Reduces Chemokine Release From Peripheral Blood Mononuclear Cells in Hyperhomocysteinemic Subjects

Kirsten B. Holven, Pål Aukrust, Torbjørn Holm, Leiv Ose, Marit S. Nenseter

Abstract—Elevated plasma homocysteine concentration is an independent risk factor for cardiovascular disease. However, the mechanisms by which hyperhomocysteinemia induces vascular disease are uncertain. An early step in atherogenesis involves leukocyte migration into the arterial wall, a process regulated in part by chemokines. We hypothesized that homocysteine may exert its atherogenic effect in part through chemokine-mediated mechanisms, and in the present study, we examined the effects of folic acid supplementation for 6 weeks on chemokine levels in hyperhomocysteinemic individuals. Data showed the following: (1) Compared with control subjects, hyperhomocysteinemic subjects had elevated plasma levels of the CXC chemokines, epithelial neutrophil-activating peptide (ENA)-78 (P<0.05), and growth-regulated oncogene (GRO)α (P=0.088), and homocysteine was significantly correlated with ENA-78 and GROα. (2) During folic acid treatment, normalization of homocysteine levels was accompanied by a marked reduction in oxidized low density lipoprotein–stimulated release of CXC chemokines (ie, GROα, ENA-78, and interleukin-8) and CC chemokines (ie, monocyte chemoattractant peptide-1 and RANTES) in peripheral blood mononuclear cells from these individuals. The oxidized low density lipoprotein–induced release of ENA-78 from peripheral blood mononuclear cells from control subjects was significantly reduced when cells were incubated in the presence of folic acid. These data may suggest that homocysteine exerts atherogenic effects in part by enhancing chemokine responses in cells involved in atherogenesis and that folic acid supplementation may downregulate these inflammatory responses. (Arterioscler Thromb Vasc Biol. 2002;22:●●●●●●.)

Key Words: homocysteine ■ folic acid ■ chemokines ■ peripheral blood mononuclear cells

An elevated plasma level of homocysteine is an independent risk factor for premature atherosclerosis and venous thromboembolic disease. Various possible mechanisms underlying the association between homocysteine and cardiovascular diseases have been suggested, eg, enhancement of oxidative stress, reduction in NO bioavailability, and augmentation of thrombus formation. However, the precise molecular mechanism is still unclear.

Recent reports have suggested a role for inflammatory processes in the pathogenesis of atherosclerosis. Thus, the migration of monocytes into the intima is a central step in the early development of atherosclerosis, and extensive infiltration of blood-derived macrophages and T cells into the vessel wall is seen in the active stages of this disorder. The recruitment of cells is mainly regulated by adhesion molecules and chemokines. Chemotactic cytokines (chemokines) are a family of inflammatory cytokines that are characterized by their ability to cause directed migration of leukocytes into inflamed tissue, but they may also have biological effects on certain other cell types, such as endothelial cells, fibroblasts, and smooth muscle cells. Chemokines have been suggested to play a pathogenic role in a number of immune-mediated disorders, and several reports have suggested that chemokines may also be involved in the pathogenesis of atherosclerosis. Thus, raised levels of chemokines, eg, interleukin (IL)-8 and monocyte chemoattractant peptide (MCP)-1, have been found in patients with atherosclerosis in the plasma and within the atherosclerotic vessel itself. Moreover, knock-out mice lacking the IL-8 (CXCR2) or the MCP-1 (CCR2) receptors have a significantly reduced progression of atherosclerosis.

On the basis of the potential pathogenic role of chemokines in atherogenesis, we hypothesized that homocysteine may exert its effect in part through chemokine-mediated mechanisms. In the present study, this hypothesis was examined by different experimental approaches.

Methods

Subjects
Twenty-six adults aged <75 years with hyperhomocysteinemia (fasting plasma total homocysteine concentration >15 μmol/L) were
TABLE 1. Characteristics of Participants

<table>
<thead>
<tr>
<th>Hyperhomocysteinemic Patients (n=26)</th>
<th>Control Subjects (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/male, n</td>
<td>7/19</td>
</tr>
<tr>
<td>Age, y</td>
<td>48 (19–73)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.1 (18.4–32.4)</td>
</tr>
<tr>
<td>CAD, n</td>
<td>4</td>
</tr>
<tr>
<td>Hypercholesterolemia, n</td>
<td>23</td>
</tr>
<tr>
<td>Statin treatment, n</td>
<td>14</td>
</tr>
<tr>
<td>Antihypertensive treatment, n</td>
<td>6</td>
</tr>
<tr>
<td>Diabetes, n</td>
<td>3</td>
</tr>
<tr>
<td>Current smokers, n</td>
<td>12</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.4 (2.7–8.7)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.3 (0.8–1.8)</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.6 (1.5–6.7)</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.2 (0.5–2.7)</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>81 (66–117)</td>
</tr>
</tbody>
</table>

n indicates number of individuals; CAD, coronary artery disease. Values are medians (ranges) or numbers of individuals.

Recruited at the Lipid Clinic, Rikshospitalet, Norway, as previously described.5 Control subjects were healthy volunteers with no history of hypertension, diabetes, coronary artery disease, or other acute or chronic illness. The protocol for the present study was approved by the Regional Committee of Medical Ethics and by The Norwegian Medicine Control Authorities. Informed consent was obtained from all subjects.

Folic Acid Treatment

The study design has been reported previously.5 Briefly, subjects with hyperhomocysteinemia received folic acid on the following schedule: 5 mg/d for the first week and 1 mg/d for the next 5 weeks. All subjects had previously been instructed by a nutritionist to follow the National Cholesterol Education Program Step I Diet, and all remained on this diet throughout the study. At baseline and after 6 weeks of treatment, venous blood samples were collected after an overnight fast. Plasma and serum samples were collected and stored at −80°C, as previously described.13

Isolation and Oxidation of LDL

Plasma from healthy blood donors, collected in endotoxin-free heparin, was stored in 0.6% sucrose at −80°C. LDL was isolated by sequential ultracentrifugation and was oxidized in the presence of freshly prepared 5 μmol/L CuSO₄ (final concentration) as described previously.18 Oxidized LDL (oxLDL), used in ex vivo experiments, contained 266 and 349 nmol/L lipid peroxides per milligram LDL protein; and relative electrophoretic mobility was 3.5 and 4.0 before and after folic acid treatment, respectively. In peripheral blood mononuclear cell (PBMC) experiments in vitro, oxLDL contained 595 ± 171 nmol/L lipid peroxides per milligram LDL protein, and relative electrophoretic mobility was 5.0 ± 0.4 (mean ± SD, n=5). LDL preparations were free of endotoxin, as determined by the Limulus assay.19 OxLDL was stored under N₂ and used within 2 weeks.

Release of Chemokines From PBMCs

PBMCs were obtained from heparinized blood by gradient centrifugation in isopaque-Ficoll (Lymphoprep, Nycomed Pharma AS) within 45 minutes and were incubated in flat-bottomed 96-well trays (2×10⁶/mL, 100 μL per well, Costar, Corning Inc) in medium alone (RPMI-1640 medium [Sigma Chemical Co] containing 2 mmol/L L-glutamine, 100 U/mL penicillin, and 5% autologous serum) or with oxLDL (final concentration 100 μg/mL). In some experiments, the cells were cultured in the presence or absence of 500 nmol/L 5-methyltetrahydrofolic acid (Sigma) in RPMI-1640 medium free of folic acid, vitamin B₁₂, and pyridoxine (GIBCO-BRL Life Technologies) supplemented with 2 mmol/L l-glutamine, 100 U/mL penicillin, and 10% of autologous serum. Cell-free supernatants were harvested after culture for 24 hours, divided into aliquots, and stored at −80°C until analysis.

Enzyme Immunoassays

Concentrations of IL-8, growth-regulated oncogene (GRO)α, epithelial neutrophil-activating peptide (ENA)-78, MCP-1, and RANTES were measured by enzyme immunoassays from R&D Systems. Concentration of IL-8 in cell-free supernatants was measured by enzyme immunoassay from CLB. To avoid run-to-run variability, all samples from a given patient were analyzed in the same microtiter plate.

Plasma Homocysteine Measurement

The plasma homocysteine concentration was determined by high-pressure liquid chromatography.20

Routine Laboratory Assays

Concentrations of folate, vitamin B₁₂, lipids, lipoproteins, and creatinine were measured in serum by using routine laboratory methods.

Statistical Analysis

Data are given as medians and ranges, unless otherwise stated. A Wilcoxon signed rank test was used to examine differences within the same individuals over time. Baseline values in patients and control subjects were compared by the Mann-Whitney U test. Spearman rank correlation coefficients were calculated to evaluate the relationships between different variables. The level of statistical significance was set at P<0.05.

Results

Characterization of Subjects

Biological characterization of the participants is shown in Table 1. Compared with healthy control subjects, the hyperhomocysteinemic patients had significantly higher homocysteine and significantly lower folate concentration (Table 2). As previously reported,17 folic acid treatment markedly increased the serum concentration of folate (≈6-fold increase, Table 2) and decreased the plasma concentration of homocysteine (≈40% decrease, Table 2). Serum concentrations of...
lipoproteins and lipids after treatment showed no significant changes from baseline (data not shown).

**Plasma Concentrations of Chemokines**

Compared with healthy volunteers, patients with hyperhomocysteinemia had elevated levels of ENA-78 (P<0.05) and GROα (P=0.088) but not IL-8, MCP-1, or RANTES (Table 2). Moreover, homocysteine concentration was positively correlated with ENA-78 (r=0.44, P=0.003) and GROα (r=0.36, P=0.02). However, although folic acid treatment markedly decreased homocysteine levels, no significant effect was seen on plasma levels of either GROα or ENA-78 or on any of the other chemokines measured (Table 2).

**Release of Chemokines From PBMCs Ex Vivo Before and After Folic Acid Treatment**

We next examined the release of chemokines from unstimulated and oxLDL-stimulated PBMCs isolated from the hyperhomocysteinemic patients with the highest homocysteine levels (median 26.1 μmol/L, range 17.9 to 52.3 μmol/L; n=12) before and after folic acid treatment. Compared with unstimulated PBMCs, oxLDL activated PBMCs to release significantly higher amounts of the CXC chemokines IL-8, GROα, and ENA-78 and the CC chemokine MCP-1 (Figure 1A through 1D). During folic acid treatment for 6 weeks, there was a normalization of plasma homocysteine levels also in this subgroup of patients (median 10.0 μmol/L, range 7.7 to 12.7 μmol/L after therapy; P=0.002 compared with baseline). Notably, this decrease in homocysteine levels was accompanied by a marked decrease in the oxLDL-stimulated release of GROα, ENA-78, MCP-1, and RANTES and, to some degree, also of IL-8 (Figure 1A through 1E). In contrast, oxLDL did not induce any release of RANTES either before or after folic acid treatment (Figure 1E). Folic acid treatment had no significant effect on unstimulated release of any of these CXC chemokines or on MCP-1, but a modest decrease in the unstimulated release of RANTES was observed (Figure 1E). There were no significant differences between statin users or nonusers either before or after folic acid treatment (data not shown). Furthermore, significant reductions in the oxLDL-stimulated release of chemokines after folic acid treatment were seen in patients with and without concomitant statin therapy, suggesting that statin users will also benefit from folic acid treatment.

**Release of Chemokines From PBMC Incubated In Vitro in the Presence or Absence of Folic Acid**

To further explore the effect of folic acid treatment on the oxLDL-stimulated release of chemokines, we measured the oxLDL-induced release of ENA-78 and GROα from PBMCs incubated in vitro in the presence or absence of folic acid. PBMCs were isolated from healthy control subjects (homocysteine levels, median 10.0 μmol/L and range 6.0 to 15.0 μmol/L; n=16). As shown in Figure 2, folic acid supplementation significantly reduced oxLDL-induced release of ENA-78, whereas no effect of folic acid was seen in unstimulated PBMCs. Moreover, although oxLDL induced an enhanced release of ENA-78 also in PBMCs from healthy control subjects, the response was markedly lower than that in patients with hyperhomocysteinemia (Figures 1 and 2). Interestingly, and in contrast to patients with hyperhomocysteinemia (Figure 1), control subjects released very low levels of GROα in unstimulated and oxLDL-stimulated cells. In fact, the release of GROα from PBMCs in the control subjects was below the detection limit in 4 of 7 subjects (14 [<10 to 604] pg/mL versus 15 [<10 to 155] pg/mL for oxLDL stimulated without and with folic acid, respectively). Accordingly, any potential effect of folic acid supplementation could not be evaluated.

**Discussion**

The present study shows the following: (1) Compared with healthy control subjects, subjects with hyperhomocysteinemia had elevated plasma levels of the CXC chemokines ENA-78 and GROα, which were significantly correlated with homocysteine levels in these subjects. (2) Most important, after folic acid treatment, normalization of homocysteine levels was accompanied by a marked reduction in the oxLDL-induced release of CXC chemokines (ie, IL-8, GROα, and ENA-78) and CC chemokines (ie, MCP-1 and RANTES) in PBMCs from hyperhomocysteinemic subjects. (3) The oxLDL-induced release of ENA-78 from PBMCs from healthy control subjects was significantly reduced when cells were cultured in vitro in the presence compared with the

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**TABLE 2. Plasma Concentrations of Homocysteine, Folate, and Chemokines in Control Subjects and in Hyperhomocysteinemic Patients Before and After 6 Weeks of Folic Acid Treatment**

<table>
<thead>
<tr>
<th></th>
<th>Hyperhomocysteinemic Patients (n=26)</th>
<th>Control Subjects (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>18.0 (8.6–52.9)*</td>
<td>9.9 (6.7–12.9)§</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.5 (8.6–12.6)</td>
</tr>
<tr>
<td>Folate, nmol/L</td>
<td>7.9 (3.9–26.7)†</td>
<td>52.5 (16.6–171.0)§</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.9 (7.3–31.1)</td>
</tr>
<tr>
<td>Vitamin B12, pmol/L</td>
<td>225 (105–830)†</td>
<td>205 (115–700)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>290 (130–510)</td>
</tr>
<tr>
<td>IL-8, pg/mL</td>
<td>&lt;10 (&lt;10–36)</td>
<td>&lt;10 (&lt;10–21)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;10 (&lt;10–13)</td>
</tr>
<tr>
<td>GROα, pg/mL</td>
<td>116 (66–574)‡</td>
<td>117 (63–547)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>89 (33–155)</td>
</tr>
<tr>
<td>ENA-78, pg/mL</td>
<td>1242 (715–5282)†</td>
<td>1166 (745–5783)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>995 (379–1848)</td>
</tr>
<tr>
<td>MCP-1, ng/mL</td>
<td>549 (225–491)‡</td>
<td>336 (152–656)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>386 (226–645)</td>
</tr>
<tr>
<td>RANTES, ng/mL</td>
<td>63 (30–200)</td>
<td>70 (32–187)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>91 (17–125)</td>
</tr>
</tbody>
</table>

Values are median (ranges).

*P<0.001 vs control subjects; †P<0.05 vs control subjects; ‡P=0.088 vs control subjects; and §P<0.001 vs before treatment.
absence of folic acid. Taken together, our data suggest that homocysteine may exert its atherogenic effect, at least in part, by enhancing inflammatory responses (ie, chemokines), which, in turn, may promote the recruitment and activation of leukocytes within the vessel wall. Moreover, in parallel with the lowering of homocysteine levels, folic acid markedly suppressed the oxLDL-stimulated release of chemokines from PBMCs, suggesting anti-inflammatory effects of folic acid therapy involving chemokine-mediated mechanisms; such effects may be beneficial in atherosclerotic patients.

Figure 1. The release of the CXC chemokines IL-8 (A), GROα (B), and ENA-78 (C) and the CC chemokines MCP-1 (D) and RANTES (E) from unstimulated (Unstim) and oxLDL-stimulated (OxLDL) PBMCs ex vivo isolated from hyperhomocysteinemic subjects before and after folic acid treatment. Compared with Unstim PBMCs, the OxLDL- PBMCs released significantly more IL-8, GROα, ENA-78, and MCP-1 before and after folic acid treatment ($P<0.005$ for all measurements). Horizontal bars represent median values. In panel E, 1 outlier is not shown.
In vitro studies have recently shown that homocysteine stimulates the expression of the CC chemokine MCP-1 in vascular smooth muscle cells, whereas homocysteine has no effect on MCP-1 production by the monocytic cell line Mono Mac 6. The present study is, to our knowledge, the first report of elevated circulating levels of CXC chemokines and of increased oxLDL-induced release of the CXC chemokine ENA-78 and GROα in PBMCs from hyperhomocysteinemic subjects compared with levels in healthy individuals. Even more important, our finding that the decrease in homocysteine levels during folic acid treatment in hyperhomocysteinemic subjects was accompanied by a marked reduction in oxLDL-induced release of CXC and CC chemokines in PBMCs from these individuals clearly suggests a relationship between hyperhomocysteinemia and enhanced chemokine levels.

Our findings with an enhanced chemokine response in hyperhomocysteinemic subjects may reflect important pathogenic processes in these individuals. Migration and activation of leukocytes is an important event in the development of atherosclerosis, and chemokines may play a crucial role in this process. Thus, enhanced IL-8 expression has been found in human coronary atheroma, and intimal macrophages with enhanced expression of the receptor for IL-8, GROα, and ENA-78 (ie, CXCR2) have been reported in murine and human atherosclerotic lesions. Also, knockout mice lacking CXCR2 or the MCP-1 receptor (CCR2) have reduced progression of atherosclerosis. Chemokines may also play an important role in plaque destabilization by directly contributing to plaque rupture and thrombus formation. Notably, it was recently reported that anti–MCP-1 directly contributing to plaque rupture and thrombus formation. GROα expression in smooth muscle cells by activating nuclear factor-κB in a concentration-dependent manner. In the present study, PBMCs were cultured in the presence of each subject’s own serum to better reflect in vivo conditions (ie, significantly higher homocysteine and lower folate concentrations at baseline compared with homocysteine and folate concentrations after folic acid treatment, suggesting a concentration-dependent effect of homocysteine). However, anti-inflammatory effects of folic acid independent of its effects on homocysteine metabolism cannot be excluded. Nevertheless, if subjects with hyperhomocysteinemia are characterized with elevated cellular secretion of chemokines in response to oxLDL also in vivo, our novel findings suggest that folic acid treatment may be an effective therapeutic approach to reduce the release of chemokines from PBMCs in these patients. Interestingly, Hofmann et al recently showed that the induction of hyperhomocysteinemia in apoE-null mice increased atherosclerotic lesion area and complexity and enhanced the expression of vascular cell adhesion molecule-1, tissue factor, matrix metalloproteinase-9, and receptors for advanced glycation end products in the vasculature. Furthermore, these homocysteine-mediated effects were significantly suppressed on supplementation with folate and vitamins B6/B12. Together with the new findings in the present study, these results suggest that dietary supplementation with folic acid and vitamins B6/B12 is beneficial in hyperhomocysteinemic patients (at least partly) by suppressing inflammatory processes. In the present study, there was a modest (not statistically significant) decline in vitamin B12 levels during folic acid treatment. Because vitamin B12 concentrations even within a “normal” range may reflect physiologically inadequate levels, forthcoming supplementation studies should most probably use folic acid in combination with vitamin B12.

Multiple epidemiological studies have indicated that elevated plasma levels of homocysteine portend an increased risk of cardiovascular disease, but the pathogenesis underlying these observations has yet to be fully defined. Although relatively few patients were studied, the present study may suggest that homocysteine exerts its atherogenic effect, at least in part, by enhancing chemokine responses in cells involved in atherogenesis. Moreover, our findings suggest that folic acid supplementation may downregulate this inappropriate response in these patients. Further studies are needed to determine whether folic acid treatment of hyper-
homocysteinemic patients is an effective therapeutic approach to prevent the progression of atherogenesis.

Acknowledgments
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References
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