Impaired Inhibitory Effect of Interleukin-10 on the Balance Between Matrix Metalloproteinase-9 and Its Inhibitor in Mononuclear Cells From Hyperhomocysteinemic Subjects

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Background and Purpose—Homocysteine has been linked to increased risk of ischemic stroke and other cardiovascular events, but the mechanism by which elevated plasma levels of homocysteine promotes atherogenesis remains unclear. Matrix degradation, partly regulated by the balance between matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), plays an important role in atherogenesis and plaque destabilization, and we hypothesized an imbalance between MMPs and TIMPs in hyperhomocysteinemia.

Methods—Serum MMP-9 and TIMP-1 was measured in 12 hyperhomocysteinemic and 12 control subjects. The release of MMP-9 and TIMP-1, with and without interleukin-10 (IL-10), and the effect of IL-10 on signal transducer and activator of transcription 3 (STAT3) phosphorylation were measured in peripheral blood mononuclear cells (PBMCs) from hyperhomocysteinemic and control subjects.

Results—Our main findings were: (1) hyperhomocysteinemic subjects had raised serum levels of MMP-9 and MMP-9/TIMP-1 ratio comparing healthy controls; (2) although IL-10 markedly suppressed MMP-9 release from PBMCs in controls, no or only minor effect was seen in hyperhomocysteinemic subjects; (3) although IL-10 enhanced TIMP-1 levels in PBMCs from both hyperhomocysteinemic and control subjects, the increase was more prominent in controls, resulting in a marked difference in IL-10–induced changes in MMP-9/TIMP-1 ratio between these 2 groups; and (4) comparing PBMCs from controls, cells from hyperhomocysteinemic individuals had impaired IL-10–induced STAT3 phosphorylation.

Conclusions—Our findings suggest an attenuated inhibitory response to IL-10 on MMP-9 activity in hyperhomocysteinemic subjects, potentially promoting atherogenesis and plaque instability, representing a novel explanation for increased risk for atherosclerotic disease in these individuals. (Stroke. 2006;37:1731-1736.)

Key Words: cytokines ■ homocysteine ■ interleukins ■ risk factors

Data from cohort and case-control studies suggest that raised homocysteine levels are associated with an increased risk of ischemic stroke and other atherosclerotic disorders, but the precise mechanism by which hyperhomocysteinemia is related to atherogenesis remains unclear. However, increasing evidence supports the involvement of inflammation in the atherosclerotic process, and we have previously shown that hyperhomocysteinemic subjects are characterized by an increased inflammatory response in peripheral blood mononuclear cells (PBMCs), possibly reflecting that homocysteine could modulate the inflammatory arm of atherosclerosis. Matrix degradation within the atherosclerotic plaque, which are modulated largely by the balance between matrix metalloproteinases (MMPs) and their endogenous inhibitors (ie, tissue inhibitors of MMPs [TIMPs]), is an important pathogenic factor in atherosclerosis, leading to enhanced migration of inflammatory cells and degradation of the fibrous cap within the lesion, promoting atherogenesis and plaque destabilization with development of acute ischemic events. Thus, the expression of some MMPs has been shown to be upregulated after cerebral ischemia, contributing to infarct extent and poor neurological outcome.

Although several inflammatory cytokines (eg, tumor necrosis factor-α [TNF-α] and interleukin-1 [IL-1]) have been found to enhance MMP activity, the anti-inflammatory cytokine IL-10 has been shown to downregulate the MMP levels accompanied by enhanced TIMP expression. Together with its ability to inhibit the release of inflammatory cytokines and tissue factor, these properties may account for the suggested antiatherogenic and plaque-stabilizing role of IL-10 in ath-
erosclerotic disorders.\textsuperscript{15–18} In humans, serum levels of IL-10 are shown to be inversely related to future events in patients with acute myocardial infarction,\textsuperscript{16} and low plasma levels of IL-10 have been associated with an early worsening in patients with subcortical infarcts or lacunar stroke.\textsuperscript{19}

Previously, we have shown that hyperhomocysteinemic subjects have elevated mRNA levels of MMP-9 and TIMP-1 in their circulating PBMCs,\textsuperscript{20} suggesting a role for altered MMP activity in the pathogenesis of hyperhomocysteine-related cardiovascular disease (CVD). The aim of the present study was to elucidate the possible mechanisms for the altered MMP-9/TIMP-1 balance in hyperhomocysteinemic subjects, particularly focusing on the role of IL-10.

**Subjects and Methods**

**Subjects**

Twelve adults \(<75\) years of age with hyperhomocysteinemia (fasting plasma total homocysteine concentration >15 \(\mu\)mol/L) were recruited at the Rikshospitalet University Hospital during 2001 to 2003. None of hyperhomocysteinemic subjects had diabetes, but 5 were treated for hypertension. Two of the subjects had previously experienced coronary artery disease (CAD), but all these had been free of symptoms for \(>6\) months. Except for CAD, none of the hyperhomocysteinemic subjects had any other concomitant inflammatory disease such as infection or autoimmune disorders. The subjects included were quite another population of individuals than those included in the intervention study performed from 1999 to 2000, analyzing MMP-9/TIMP-1 gene expression in PBMCs,\textsuperscript{20} with no overlap between the 2 study populations. Control subjects were 12 sex- and age-matched healthy volunteers with no history of hypertension, diabetes, CVD, or other acute or chronic illness. The study protocol was approved by the regional committee of medical ethics. Informed consent was obtained from all subjects. Plasma and serum samples were collected after an overnight fast and stored at \(-80^\circ\)C until analysis.

**Cell Isolation and Culturing**

PBMCs, obtained from heparinized blood by gradient centrifugation in Isopaque-Ficoll (Lymphoprep; Nycomed), were incubated for various time points in flat-bottomed 96-well trays (Costar; \(2 \times 10^6/\)mL) in medium (RPMI-1640 medium [Sigma Chemical] containing 2 \(\mathrm{mmol/L}\) L-glutamine, and 5\% autologous serum) with or without IL-10 (10 ng/mL; R&DSystems). Cell-free supernatants and cell pellets were harvested after various time points and stored at \(-80^\circ\)C. Because a relatively large number of cells were necessary in some of the experiments, we were not able to perform all analyses in all individuals.

**Ribonuclease Protection Assay**

Total RNA was extracted from PBMCs using RNeasy columns (Qiagen) and stored in RNA storage solution (Ambion) at \(-80^\circ\)C. Ribonuclease protection assay was performed using the bCr-3b multiprobe (Pharmingen).\textsuperscript{22} The mRNA signal was normalized to the signal from the housekeeping genes rpL32 and GADPH.

**Western Immunoblotting**

Western blot analysis was performed as described previously\textsuperscript{23} with separation on SDS/7.5\% PAGE (Bio-Rad) of equal amounts of protein (10 \(\mu\)g) before transfer to polyvinylidene difluoride filters (NEN; Life Science). After block for 1 hour, the filters were incubated with either the anti-human signal transducer and activator of transcription 3 (STAT3) or the anti-human Janus family tyrosine kinase 1 (JAK1) or phosphospecific anti-human STAT3 or JAK1 (Cell Signaling). By reprobing the blots with unphosphorylated antibodies, we ensured equal loading. The immune complex was detected by Supersignal West Pico Western blot detection (Pierce).\textsuperscript{23}

**Enzyme Immunoassays**

Concentrations of MMP-9, TIMP-1, IL-8, IL-10, and TNF-\(\alpha\) were measured by enzyme immunoassays (R & D Systems).

**Routine Laboratory Assays**

Concentrations of homocysteine, folate, vitamin B\(_12\), and other indicated routine laboratory assays were measured by routine laboratory methods.\textsuperscript{24}

**Statistical Analysis**

Data are given as mean \(\pm\) SD or median (minimum–maximum) if not otherwise stated. Data from patients and controls were compared by Student \(t\) test or by the Mann–Whitney \(U\) test as appropriate. Spearman rank correlation coefficients were calculated to evaluate relationships between different variables. \(P\) values (2-sided) were considered significant at values of \(\leq0.05\).

**Characterization of Subjects**

Compared with healthy controls, the hyperhomocysteinemic subjects had significantly higher homocysteine and significantly lower folate and vitamin B\(_12\) concentrations, as well as significantly raised levels of C-reactive protein and total leukocyte counts (Table).

**Serum Concentrations of MMP-9 and TIMP-1**

Compared with healthy controls, hyperhomocysteinemic subjects had elevated serum levels of MMP-9, with no differences in TIMP-1, resulting in a significantly higher MMP-9/TIMP-1 ratio in those with hyperhomocysteinemia (Figure 1).

**Characteristics of Participants**

\[
\begin{array}{cccc}
\text{Hyperhomocysteinemic} & \text{Control Subjects} \\
\text{Subjects} & (n=12) & (n=12) \\
\text{Age, y} & 51.5 (25–58) & 51.5 (25–58) \\
\text{Male, n} & 7 & 7 \\
\text{CAD, n} & 2 & 0 \\
\text{Statin treatment, n} & 3 & 0 \\
\text{Current smokers, n} & 8 & 0 \\
\text{Body mass index, kg/m}^2 & 24.1 (19.6–39.9) & 24.3 (21.8–28.7) \\
\text{Homocysteine, \mu mol/L} & 29 (19–75)*** & 11 (7–14) \\
\text{Folate, \mu mol/L} & 5.4 (3.3–8.8)*** & 14.6 (5.4–30.3) \\
\text{Vitamin B\(_12\), \mu mol/L} & 200 (110–325) & 258 (120–440) \\
\text{Cholesterol, mmol/L} & 5.8 (3.9–7.8) & 6.4 (6.0–6.7) \\
\text{High-density lipoprotein cholesterol, mmol/L} & 1.3 (1.0–1.5) & 1.4 (0.9–2.3) \\
\text{Low-density lipoprotein cholesterol, mmol/L} & 3.8 (1.7–6.0) & 3.7 (2.9–4.4) \\
\text{Triglycerides, mmol/L} & 1.3 (0.5–2.9) & 1.0 (0.4–2.9) \\
\text{Glucose, mmol/L} & 5.3 (4.7–6.7) & 5.1 (4.1–6.1) \\
\text{Creatinine, \mu mol/L} & 78 (66–98) & 86 (72–104) \\
\text{C-reactive protein, mg/L} & 2.2 (0.4–12.0)* & 0.7 (0.2–3.5) \\
\text{Leukocytes, 10\(^6/\)L} & 6.2 (4.8–9.5)* & 5.1 (3.7–7.7) \\
\text{Lymphocytes, 10\(^6/\)L} & 2.0 (0.7–4.4) & 1.6 (1.1–2.9) \\
\text{Monocytes, 10\(^6/\)L} & 0.43 (0.30–0.70) & 0.41 (0.28–0.58)
\end{array}
\]

Data are given as median (minimum–maximum); \(n\) indicates No. of individuals.

*\(P<0.05\) and **\(P<0.001\) vs control subjects.
Release of MMP-9 and TIMP-1 From PBMCs Ex Vivo
We have previously shown enhanced gene expression of MMP-9 and TIMP-1 in PBMCs from hyperhomocysteinemic individuals, and because IL-10 is known to modulate these matrix-regulating mediators, we next examined the effect of IL-10 on the release of MMP-9 and TIMP-1 in PBMCs from the 12 hyperhomocysteinemic individuals and the 12 controls after culturing for 24 hours. Although IL-10 markedly suppressed the spontaneous release of MMP-9 from PBMCs in healthy controls, no or only minor effect was seen in cells from hyperhomocysteinemic subjects (Figure 2). Although IL-10 enhanced TIMP-1 levels in both hyperhomocysteinemic and control subjects, the increase was more prominent in healthy controls, resulting in a marked difference in the IL-10-induced changes in the MMP-9/TIMP-1 ratio between these 2 groups of individuals (Figure 2). In contrast, there were no differences in IL-10-mediated inhibition of IL-8 and
TNF-α release between PBMCs from hyperhomocysteinemic and control subjects (data not shown).

**Serum Levels of IL-10**

We have previously reported raised levels of various inflammatory mediators in individuals with hyperhomocysteinemia. However, no such increase was found for IL-10 showing similar concentrations in hyperhomocysteinemic (1.0 pg/mL [0.3 to 4.2 pg/mL]; n=12) and control subjects (0.9 pg/mL [0.1 to 1.6 pg/mL]; n=12), suggesting inflammatory net effects in those with hyperhomocysteinemia. Moreover, as a reflection of the raised MMP-9 accompanied by normal IL-10 levels, these mediators were inversely correlated in the hyperhomocysteinemic individuals ($r=-0.64; P=0.035$).

**IL-10 Receptors in PBMCs**

To elucidate the mechanisms for the impaired IL-10 response in PBMCs from hyperhomocysteinemic individuals, we first examined the gene expression of IL-10 receptors (IL-10R) in PBMCs from 8 randomly selected hyperhomocysteinemic subjects and 8 matched controls. Notably, although the hyperhomocysteinemic individuals showed impaired IL-10 response compared with controls, there was no significant difference in mRNA levels of IL-10R or IL-10R2 (subunit interacting to assemble the active IL-10R complex) between these groups (Figure 3).

**STAT Signaling Pathway**

The anti-inflammatory effect of IL-10 has been suggested to be mediated via activated STAT3. To further explore the mechanisms of the impaired IL-10–mediated effect on MMP-9 and TIMP-1 in PBMCs from hyperhomocysteinemic subjects, we analyzed the IL-10–mediated effects on the STAT signaling pathway. When comparing STAT3 phosphorylation in PBMCs from the 12 hyperhomocysteinemic and the 12 matched control subjects after 24 hours, representing the steady-state situation, we found a significantly lower proportion of IL-10–mediated STAT3 phosphorylation, with no differences in unstimulated STAT3 phosphorylation, in cells from hyperhomocysteinemic individuals (Figure 4). In contrast, we found that the degree of IL-10–mediated JAK1 phosphorylation in PBMCs from hyperhomocysteinemic individuals (n=4) was within the range of those values that was found in PBMCs from healthy controls (n=4; data not shown).

**MMP-9, TIMP-1, and IL-10 Levels in Relation to Clinical Characteristics**

We found no significant difference between hyperhomocysteinemic smokers (n=8) and nonsmokers (n=4) or between hyperhomocysteinemic statin users (n=3) and nonusers (n=9) in any of the parameters tested (ie, serum MMP-9, TIMP-1, IL-10, release of MMP-9 from PBMCs with or without IL-10, and IL-10–mediated STAT-3 phosphorylation). These findings suggest that our data in the hyperhomocysteinemic individuals do not merely reflect that there were more smokers in the hyperhomocysteine group; there were only 2 individuals with CAD among the hyperhomocysteinemic subjects, and when they were excluded from the analysis, the same pattern was observed.

**Discussion**

Matrix degradation has been suggested to play an important role in atherogenesis and plaque destabilization, and has also been shown to be involved in tissue injury counteracting the benefit of thrombolytic therapy in the hyperacute phase of stroke. Herein we show that hyperhomocysteinemic subjects have an elevated MMP-9/TIMP-1 ratio in serum, suggesting matrix-degrading net effects. Moreover, we show a markedly impaired ability of IL-10 to suppress MMP-9 and enhance TIMP-1 levels in PBMCs from hyperhomocysteinemic subject, possibly involving impaired IL-10–mediated STAT3 phosphorylation. Our findings suggest an attenuated inhibitory response to IL-10 on MMP activity in hyperhomo-
cysteimic individuals, possibly representing a novel explanation for the increased risk of cardiovascular events in these individuals.

It has been reported that homocysteine may induce MMP-9 secretion from endothelial cells in vitro, and studies in animal models suggest that this MMP-9–inducing effect may contribute to the endothelial-damaging properties of homocysteine. We previously reported enhanced MMP-9 mRNA levels in PBMCs of homocysteimic patients showing a significant reduction during folic acid therapy. Herein, we extend these findings by demonstrating raised serum levels of MMP-9 in hyperhomocysteimic patients, accompanied by no changes in TIMP-1, suggesting matrix degrading net effects. Serum MMP-9 concentrations have been reported to be a predictor of cardiovascular mortality in patients with CVD, and several lines of evidence suggest that enhanced MMP activity may be involved in atherogenesis and plaque rupture. Our findings may suggest that such mechanisms also may be operating in homocysteimic patients, possibly contributing to the increased risk for stroke and cardiovascular events in these individuals.

In contrast to the findings in PBMCs from healthy controls, we found no IL-10–mediated suppression of MMP-9 and an attenuated IL-10–mediated increase of TIMP-1 in PBMCs from hyperhomocysteimic subjects, resulting in only a modest decrease in MMP-9/TIMP-1 ratio after IL-10 stimulation in those with hyperhomocysteine. We have previously shown that IL-10 suppresses MMP-9 and enhances TIMP-1 levels in CAD patients, but in that study, none of the patients had raised homocysteine levels, and our findings in the present study suggest that these apparently beneficial effects of IL-10 in CAD patients may be counteracted in those with accompanying hyperhomocysteine. This potential lack of matrix stabilizing effects of IL-10 in PBMCs from hyperhomocysteimic subjects could contribute to the raised MMP-9/TIMP-1 ratio in these individuals.

STAT3 has been shown to be of importance for the anti-inflammatory effects of IL-10. Moreover, TIMP-1 has been reported to be a STAT3 target gene in various cell types. It is therefore conceivable that the impaired IL-10–mediated STAT3 phosphorylation could contribute to the attenuated effect of IL-10 on the MMP-9/TIMP-1 levels in PBMCs from hyperhomocysteimic individuals. However, other anti-inflammatory effects (eg, inhibition of IL-8 and TNF-α), possibly also related to STAT3 phosphorylation, was not impaired in those with hyperhomocysteine. The reason for this selective impairment of the IL-10–mediated effects on MMP-9/TIMP-1 is presently unclear but could reflect STAT3-independent anti-inflammatory effects of IL-10 or that the inhibition of STAT3 phosphorylation is counteracted by other homocysteine-mediated effects on the intracellular signaling pathways.

The mechanisms by which persistently raised homocysteine levels inhibit IL-10–mediated STAT3 phosphorylation are presently unknown. Although the lack of effect on IL-10–mediated JAK1 phosphorylation may suggest that homocysteine is operating downstream for this kinase, our data on the JAK1/STAT3 signaling pathway must be interpreted with caution. First, the degree of JAK1 phosphorylation was only examined in a few individuals. Second, the decreased IL-10–induced STAT3 phosphorylation in hyperhomocysteimic individuals may be secondary to increased activation of other kinases acting upstream for STAT3 (eg, Tyk2). Finally, the degree of STAT3 phosphorylation may also be modulated by IL-10–independent mechanisms, and the mechanisms for the impaired IL-10–mediated STAT3 phosphorylation in PBMCs from hyperhomocysteimic individuals should be further investigated in forthcoming studies.

The present study shows an altered MMP-9/TIMP-1 balance in hyperhomocysteimic individuals, potentially resulting in a matrix-degrading phenotype that may contribute to the increased risk of ischemic stroke and other CVDs in these subjects. Moreover, we show an attenuated suppressive effect of IL-10 on the MMP-9/TIMP-1 levels in PBMCs from hyperhomocysteimic individuals, possibly involving impaired IL-10–mediated STAT3 phosphorylation. Although relatively few individuals were studied and although we lack mechanistic data to explain the attenuated IL-10 effect, the decreased IL-10–mediated STAT3 phosphorylation in PBMCs from hyperhomocysteimic individuals, as well as the raised MMP-9/TIMP-1 levels in these subjects, should be further investigated as potential important mechanisms for the proatherogenic effects of homocysteine.

Acknowledgments
We thank Azita Rashidi and Ellen Lund Sagen for excellent technical assistance.

Sources of Funding
This work was supported by grants from the Norwegian Foundation for Health and Rehabilitation, the Norwegian Association of Heart and Lung Patients, the Norwegian Council on Cardiovascular Disease, and from the University of Oslo, Norway.

Disclosures
None.

References


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*Stroke*. 2006;37:1731-1736; originally published online May 25, 2006; doi: 10.1161/01.STR.0000226465.84561.cb

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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