Hyperhomocysteinemia Promotes Inflammatory Monocyte Generation and Accelerates Atherosclerosis in Transgenic Cystathionine β-Synthase–Deficient Mice

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Background—Hyperhomocysteinemia (HHcy) is an independent risk factor for cardiovascular disease. Monocytes display inflammatory and resident subsets and commit to specific functions in atherogenesis. In this study, we examined the hypothesis that HHcy modulates monocyte heterogeneity and leads to atherosclerosis.

Methods and Results—We established a novel atherosclerosis-susceptible mouse model with both severe HHcy and hypercholesterolemia in which the mouse cystathionine β-synthase (CBS) and apolipoprotein E (apoE) genes are deficient and an inducible human CBS transgene is introduced to circumvent the neonatal lethality of the CBS deficiency (Tg-hCBS apoE−/− Cbs−/− mice). Severe HHcy accelerated atherosclerosis and inflammatory monocyte/macrophage accumulation in lesions and increased plasma tumor necrosis factor-α and monocyte chemoattractant protein-1 levels in Tg-hCBS apoE−/− Cbs−/− mice fed a high-fat diet. Furthermore, we characterized monocyte heterogeneity in Tg-hCBS apoE+/+ Cbs−/− mice and another severe HHcy mouse model (Tg-S466L Cbs−/−) with a disease-relevant mutation (Tg-S466L) that lacks hyperlipidemia. HHcy increased monocyte population and selective expansion of inflammatory Ly-6Chi and Ly-6Cmid monocyte subsets in blood, spleen, and bone marrow of Tg-S466L Cbs−/− and Tg-hCBS apoE−/− Cbs−/− mice. These changes were exacerbated in Tg-S466L Cbs−/− mice with aging. Addition of L-homocysteine (100 to 500 μmol/L), but not L-cysteine, maintained the Ly-6Chi subset and induced the Ly-6Cmid subset in cultured mouse primary splenocytes. Homocysteine-induced differentiation of the Ly-6Cmid subset was prevented by catalase plus superoxide dismutase and the NAD(P)H oxidase inhibitor apocynin.

Conclusion—HHcy promotes differentiation of inflammatory monocyte subsets and their accumulation in atherosclerotic lesions via NAD(P)H oxidase–mediated oxidant stress. (Circulation. 2009;120:1893-1902.)

Key Words: atherosclerosis ■ hyperhomocysteinemia ■ inflammation ■ leukocytes

Despite advances in our understanding of the pathogenesis of cardiovascular disease (CVD), the established risk factors do not fully account for its occurrence. Abundant clinical and epidemiological studies have revealed the close relationship between hyperhomocysteinemia (HHcy) and the increased risk of CVD by 1.6- to 1.8-fold, which is similar to the risk seen with an increase of 20 mg/dL (0.52 mmol/L) in cholesterol concentration. Therefore, HHcy has been identified as a potent risk factor for CVD, equivalent to smoking and hyperlipidemia.

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During the last decade, profound atherogenic effects of HHcy have been established in experimental models. We and others have demonstrated that HHcy promotes atherogenesis by stimulating vascular smooth muscle cell proliferation, inhibiting endothelial cell growth and postinjury reendothelialization, impairing endothelial relaxation, accelerating neointimal formation, and inhibiting high-density lipoprotein biosynthesis. It has been suggested that the autoimmune response induced by HHcy may be intimately related to CVD. However, the fundamental basis of HHcy-induced immune response and its contribution to atherosclerosis remain unknown.

Support for atherosclerosis as a systemic inflammatory process has recently grown significantly. Monocytes migrate from the bloodstream and give rise to tissue macrophages and dendritic cells. Macrophages accumulate in the vessel wall, take up modified lipoprotein, and participate in atherogenesis.
We now know that circulating monocytes, in both humans and mice, are heterogeneous and that different monocyte subsets exhibit distinct pathophysiological roles. The inflammatory monocyte subset is recruited into inflammatory tissues; the resident monocyte subset enters normal tissues. Emerging new evidence supports the notion that monocyte heterogeneity bridges the pathogenic mechanism of dyslipidemia and the inflammatory response in the progression of atherosclerosis. How HHCy influences monocyte heterogeneity has not been investigated.

Cystathionine β-synthase (CBS) is a key enzyme in the transsulfuration pathway that catalyzes Hcy. The S466L mutation of the CBS gene was identified in homocystinuric patients and is associated with thrombosis and elevated levels of plasma Hcy (≈167 μmol/L). These findings support the clinical applicability of using genetic animal models to study the effect of HHCy on CVD.

Maeda and colleagues created a mouse model for Cbs−/− in which mice have plasma Hcy levels of ≈200 μmol/L. However, most Cbs−/− mice have a short life span and die before weaning. We have generated apoE−/− Cbs−/− mice by cross-breeding apoE−/− mice with Cbs−/− mice and are the first to demonstrate that severe HHCy accelerates atherosclerosis independently of dietary manipulation in apoE−/− Cbs−/− mice. These studies were difficult and relied on breeding a large colony of mice. To circumvent the neonatal lethality problem in the Cbs−/− mice, the Kruger laboratory created a transgenic mouse (Tg-hCBS) in which the human CBS cDNA is under the control of a zinc-inducible metallothionein promoter. By crossing these animals to Cbs−/− mice and supplying zinc in the drinking water during pregnancy and lactation, Tg-hCBS was able to rescue the neonatal lethal phenotype and generated Tg-hCBS Cbs−/− animals. At weaning, zinc was removed and the expression of Tg-hCBS was stopped, which caused the animals to develop severe HHcy (plasma Hcy >150 μmol/L). A second mouse model was developed in which a mutant human CBS transgene (Tg−S466L) was used in a similar manner. In this study, we also generated an atherosclerosis-susceptible HHCy model (Tg−hCBS apoE−/− Cbs−/−). Using these newly established models of severe HHcy, we report here our findings from tests of the novel hypothesis that HHcy modulates monocyte heterogeneity and accelerates atherogenesis.

Methods

Gene-Targeted Mice and Diet

All mice are in a C57BL/6 strain background. The transgenic mice, Tg−hCBS and Tg−S466L, were established as described previously. Pups were genotyped at day 10 for both the human transgene and the mouse CBS deficiency as previously described. The Tg−hCBS apoE−/− Cbs−/− mouse line was generated by crossing Tg−hCBS Cbs−/− mice with apoE−/− mice (The Jackson Laboratory, Bar Harbor, Me). These animals were all born to mothers drinking zinc water to induce transgene expression. ZnCl2 was withdrawn after weaning at 1 month of age.

Animals were fed standard rodent chow diet (0.43% methionine; TD 2018SX; Harlan Teklad, Madison, Wis) before dietary intervention. Age-matched littermates with evenly distributed sex were selected for each experimental group. Control mice for all experiments were sibling transgene-positive mice that were in either Cbs−/− or Cbs−/+ background. The Cbs−/− and Cbs−/+ mice in the control groups have similar plasma Hcy levels (data not shown). This might be related to the human CBS transgene, which provides partial function to metabolize Hcy in both lines. For lesion analysis, Tg−hCBS apoE−/− Cbs−/− mice and their sibling controls were switched to a high-fat (HF) diet (0.2% cholesterol, 21.2% fat, 0.75% methionine, 1.43 mg/g choline, 2.23 mg/kg folate, 32.5 μg/kg B12, 22.25 mg/kg B6; TD8137; Harlan Teklad) at 8 weeks of age and were maintained on this diet for an additional 8 weeks. The mouse protocols were approved by the Temple University Institutional Animal Care and Use Committee.

Mouse Primary Splenocyte Culture and Chemical Treatments

Mouse primary splenocytes from 2-month-old wild-type mice (C57Bl/6) were isolated as described in the Methods section of the online-only Data Supplement. Splenocyte suspensions were plated at a density of 1.5×106 cells per well in 24-well plates in RPMI1640 medium (HyClone, Logan, Utah) supplemented with 10% FCS, 2 mmol/L l-glutamine, 10 mmol/L HEPES, 0.1 mmol/L nonessential amino acid, 1 mmol/L sodium pyruvate, 50 μmol/L 2-mercaptopethanol, and 1% antibiotics (penicillin, streptomycin). To examine the effect of Hcy on Ly-6C monocyte subset survival, cells were treated with L-Hcy (L-Hcy; 100 to 500 μmol/L), L-cysteine (L-Cys; 100 to 500 μmol/L) as the sulfhydryl-containing amino acid control, or recombinant mouse interferon-γ (100 U/mL, R&D Systems Inc, Minneapolis, Minn) as a classic inducer of Ly-6C expression at 0 hour and continued for 24 hours. To determine the effect of Hcy on Ly-6C monocyte subset differentiation, splenocytes were treated with L-Hcy or L-Cys at 24 hours after plating for an additional 48 hours.

To illustrate the mechanism mediating HHcy-promoted inflammatory phenotypic shift in monocytes, splenocytes were cultured for 24 hours and then treated with the antioxidants catalase-polypolyene glycerol (CAT-PEG; 250 U/mL) plus superoxide dismutase-PEG (SOD-PEG; 250 U/mL), the peroxynitrite inhibitor uric acid (60 μg/mL), the NAD(P)H oxidase inhibitor apocynin (100 μmol/L), the xanthine oxidase inhibitor allopurinol (30 μg/mL), or the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 1 mmol/L) 1 hour before exposure to L-Hcy (500 μmol/L). To test the hypomethylation mechanism, cells were treated with 50 μmol/L L-Hcy in the presence of 25 μmol/L adenine and 10 μmol/L cytidine-9-(2-hydroxy-3-nonyl)-adenine hydrochloride, an adenosine deaminase inhibitor, to stabilize adenosine as we reported previously. Adenosine is a normal constituent of all body fluids and is not stable in the cell culture medium. The addition of adenosine and its stabilizer facilitates the conversion of Hcy to S-adenosylhomocysteine, a potent inhibitor of methyltransferase, and sensitizes a hypomethylation mechanism.

L-Hcy was freshly prepared by reducing L-homocysteine with a 2-fold molar excess of dithiothreitol for 30 minutes at 37°C, pH 8.0, as described. All chemicals, if not specified above, were purchased from Sigma-Aldrich (St Louis, Mo).

The following are described in the Methods section of the online-only Data Supplement: body and organ weight measurements; gene expression analysis; monocyte subset counting; flow cytometry analysis; and statistical analysis.

Results

Severe HHcy Reduced Body Weight in Tg-hCBS apoE−/− Cbs−/− Mice

In Tg−S466L Cbs−/− mice at 6 months of age, plasma Hcy was 213.9±67.8 μmol/L and was not changed at 8 months of age. In contrast, control animals had plasma Hcy of 4.5 μmol/L at 6 months and 4.1 μmol/L at 8 months of age (Figure 1A). Methionine levels were slightly elevated in Tg−S466L Cbs−/− mice and slightly increased with age (56.6±13.9 versus...
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42.3±1.2 μmol/L at 6 months and 68.2±5 versus 47.9±11.3 μmol/L in the controls at 8 months of age). Hcy levels in Tg-hCBS apoE−/− Cbs−/− mice fed a HF diet were increased to 179.5±24.1 μmol/L (P<0.001; Figure 1B). In this strain, methionine levels were not significantly different from that in the control mice. In Tg-hCBS apoE−/− Cbs−/− mice fed a regular diet, plasma levels of Hcy and methionine were increased to 200.7±19.4 μmol/L (P<0.001) and 107.7±16.1 μmol/L (P=0.004), respectively (Figure 1C).

In Tg-hCBS apoE−/− Cbs−/− mice fed an HF diet, severe HHcy was associated with a 15.6% reduction in body weight (P=0.04; Figure 1D). However, the weights of heart, liver, and kidney (Figure 1E, 1F, and 1H) were not changed in Tg-hCBS apoE−/− Cbs−/− mice. The trend of a decrease in spleen weight did not reach statistical significance (Figure 1G).

**Severe HHcy Accelerated Atherosclerosis and Enhanced Ly-6C Monocyte/Macrophage Accumulation in Lesions in Tg-hCBS apoE−/− Cbs−/− Mice**

To confirm the atherogenic effects of HHcy, Tg-hCBS apoE−/− Cbs−/− and their sibling control mice were fed a HF diet and examined for atherosclerotic lesions by cross-sectional analysis of the aortic sinus (Figure 2A). Atherosclerotic lesions were significantly increased in Tg-hCBS apoE−/− Cbs−/− mice (63.8±14.6×10² μm²; P=0.004) compared with those of their sibling controls (39.2±15×10² μm²). The lesion percentage of aortic sinus was increased from 29.7±8.7% in the controls to 39.7±6.5% (P=0.019) in Tg-hCBS apoE−/− Cbs−/− mice. The atherosclerotic lesion areas were positively correlated with plasma Hcy levels (r=0.620, P=0.008; Figure 2C).

We further examined the involvement of monocytes in HHcy-related atherosclerosis. Aortic sinus cross sections were stained with MOMA-2 antibody, a marker for monocyte/macrophage, and Ly-6C, a marker for the inflammatory monocyte subset (Figure 2B). Monocyte/macrophage area was significantly increased in the lesions of Tg-hCBS apoE−/− Cbs−/− mice (47.7±7.6×10⁴ μm²; P<0.001) compared with their controls (18.5±10.7×10⁴ μm²). The percentage of monocyte/macrophage area in the lesion was increased from 46.9±20.7% in the controls to 76.3±12.3% (P=0.005) in Tg-hCBS apoE−/− Cbs−/− mice. Lesion monocyte/macrophage area was positively correlated with plasma Hcy levels (r=0.687, P=0.003; Figure 2D).

The Ly-6C–positive area (23.6±4.6×10⁴ μm²; P=0.001) and the percentage of Ly-6C–positive area in the lesion (38.7±11.4%; P<0.001) were elevated in Tg-hCBS apoE−/− Cbs−/− mice compared with their controls (6.8±4.1×10⁴ μm² and 17.9±5.1%). Ly-6C–positive areas were positively related to plasma Hcy levels (r=0.722, P=0.002). The Ly-6C and MOMA-2 double-positive areas represented the Ly-6C inflammatory monocyte/macrophage subset, which was increased in the lesion of Tg-hCBS apoE−/− Cbs−/− mice (10.2±3.8×10⁴ μm² and 17.5±8.4% of the lesion; P<0.001 and P=0.004) compared with their controls (1.6±0.8×10⁴ μm² and 4.8±1.9% of the lesion). Ly-6C–positive monocyte/macrophage accumulation in the lesion was positively correlated with Hcy levels (r=0.678, P=0.005; Figure 2F).

**Severe HHcy Increased Plasma TNF-α and MCP-1 Levels in Tg-hCBS apoE−/− Cbs−/− Mice**

Severe HHcy increased plasma levels of the proinflammatory cytokine TNF-α (182.8±23.39 versus 146.7±9.36 pg/mL; P=0.007) and the chemotactic factor MCP-1 (682.9±320.32 versus 265.9±50.14 pg/mL; P=0.011) in Tg-hCBS apoE−/− Cbs−/− mice after 8 weeks of HF diet compared with their sibling controls on the same diet. Furthermore, we identified significant positive correlations between the concentrations of TNF-α (r=0.706, P=0.005) and MCP-1 (r=0.745, P=0.008) and Hcy levels in these mice (Figure 3).

**Severe HHcy Increased the Monocyte Population in Both Tg-S466L Cbs and Tg-hCBS apoE−/− Cbs Mice**

We studied the BM and spleen because BM produces monocytes and spleen serves as a reservoir for circulating monocytes. As shown in Figure 4A, mononuclear cells (MNCs) were gated in gate ii on the basis of granularity and cell size because of their lower granular content and relatively larger size compared with granulocytes and lymphocytes. The

![Figure 1. Plasma levels of Hcy and methionine (Met) and organ weight in transgenic CBS-deficient mice. Tg-hCBS apoE−/− Cbs mice were fed a HF diet at 8 weeks of age for an additional 8 weeks. The Tg-S466L Cbs and Tg-hCBS apoE−/− Cbs mice were fed a rodent chow. A through C, Plasma levels of Hcy and Met, D, Body weight, E through H, Heart, liver, spleen, and kidney weights relative to tibia lengths, respectively. Values are mean±SD, P values are from independent t tests. n=10 to 12.](http://circ.ahajournals.org/content/189/19/1895)
gate ii MNCs were then further grouped on the basis of the expression levels of CD11b, a monocyte marker. Monocytes were defined as CD11b^+ MNCs and described in the histogram of Figure 4A. The percentage of CD11b^+ MNCs or monocytes in nucleated cells (gate i) is presented in Figure 4B. Severe HHcy significantly increased the monocyte population in blood, spleen, and BM in 6-month-old Tg-S466L Cbs^+/− mice. Interestingly, HHcy-induced monocyte expansion appeared to be enhanced with aging; monocyte populations were further increased in all 3 tissues isolated from 8-month-old Tg-S466L Cbs^+/− mice.

In addition, we examined the monocyte population in 15-month-old Tg-hCBS apoE^−/− Cbs^+/− mice without dietary manipulation (Figure 4C and 4D). Severe HHcy significantly promoted monocyte expansion in blood, spleen, and BM in Tg-hCBS apoE^−/− Cbs^+/− mice.

Severe HHcy Elevated CD11b^+Ly-6C^hi and CD11b^+Ly-6C^mid Inflammatory Monocyte Subsets in Both Tg-S466L Cbs and Tg-hCBS apoE^−/− Cbs Mice

We further characterized monocyte subsets by flow cytometry analysis using anti-CD11b and anti-Ly-6C antibodies. Monocytes expressing high levels of Ly-6C have previously been shown to represent the inflammatory subset. CD11b^+ MNCs within gate ii, defined in Figure 4, were further divided into 3 groups based on their Ly-6C expression levels (low, middle, and high; Figure 5A). The CD11b^+Ly-6C^hi inflammatory monocyte subset was the most abundant group in blood and increased with age in all 3 tissues. Tg-S466L Cbs^+/− mice at 6 months of age, compared with their controls, had increased percentages of CD11b^+Ly-6C^hi cells in blood (42.3±6.9% versus 22.4±10.4%; P=0.019), spleen (5.4±1.5% versus 3.1±0.7%; P=0.01), and BM (12.9±1.6% versus 6.4±0.8%; P=0.001). This was further increased in blood and spleen and stabilized in BM at 8 months of age. The CD11b^+Ly-6C^mid monocytes were increased in all 3 tissues in Tg-S466L Cbs^+/− mice at 6 months of age, compared with their controls, had increased percentages of CD11b^+Ly-6C^mid cells in blood (42.3±6.9% versus 22.4±10.4%; P=0.019), spleen (5.4±1.5% versus 3.1±0.7%; P=0.01), and BM (12.9±1.6% versus 6.4±0.8%; P=0.001). This difference disappeared at 8 months of age in all 3 tissues. The CD11b^+Ly-6C^low resident monocyte subset in the spleen and in the BM increased between 6 and 8 months of age in both Tg-S466L Cbs^+/− and control animals. In blood and

Figure 2. HHcy accelerated atherosclerotic lesions and enhanced monocytes/macrophages (MC/MΦ) and Ly-6C^−/−MC/MΦ accumulation in the lesion in Tg-hCBS apoE^−/− Cbs mice. Mice were fed a HF diet at 8 weeks of age for an additional 8 weeks. A, Photomicrographs of mouse aortic sinus cross sections stained with Oil Red O and counterstained with hematoxylin. B, Photomicrographs of mouse aortic sinus cross sections immunostained with MOMA-2 (MC/MΦ marker; green), Ly-6C (red), and DAPI (blue). Merge (yellow to orange) shows the accumulation of Ly-6C^−/−MC/MΦ in the lesion. C through F, Quantitative analysis of lesions in the aortic sinuses. Atherosclerotic lesion area was defined as the neointimal region between the lumen and internal elastic lumina (IEL) (C), MC/MΦ area (D), Ly-6C^−/−area (E), and Ly-6C^−/−MC/MΦ area (F). Correlation analyses of Hcy levels with lesion area, MC/MΦ area, Ly-6C^−/−area, and Ly-6C^−/−MC/MΦ area are shown in scattered dot graphs. Each data point represents 1 mouse. Values are mean±SD. n=8 to 9.

To assess monocyte differentiation, we treated splenocytes with L-Hcy or L-Cys at 24 hours after plating for an additional 48 hours and then examined Ly-6C expression, as others described in BM cells and monocytic cell lines.\textsuperscript{22,23} We found that the CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid} subset was significantly reduced with culturing time in the controls. The CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid} subset made up 15.9\% of the MNCs when freshly isolated (0 hour), which was reduced to 10.6\% and 8.7\% after 24 and 72 hours of culture, respectively. L-Hcy (100, 200, and 500 \textmu M) significantly increased the CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid} subset after 72 hours of culture in a dose-dependent manner, from 8.7\% (control) to 11.2\% (P<0.001), 12.2\% (P<0.001), and 15\% (P<0.001), respectively. In contrast, L-Cys did not change the proportions of CD11b\textsuperscript{+} Ly-6C\textsuperscript{hi} and CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid}. The significant differences between L-Hcy and L-Cys treatment on CD11b\textsuperscript{+} Ly-6C\textsuperscript{hi} and CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid} subset differentiation confirmed the specific effects of Hcy. Finally, both L-Hcy and L-Cys did not change the CD11b\textsuperscript{+} Ly-6C\textsuperscript{low} subset after 72 hours in culture.

Hcy-Induced CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid} Subset Formation Was Prevented by CAT Plus SOD and an NAD(P)H Oxidase Inhibitor in Cultured Mouse Primary Spleenocytes

To elucidate the mechanism underlying Hcy-induced inflammatory monocyte differentiation, we preincubated mouse splenocytes with various inhibitors or chemicals before Hcy treatment (Figure 7A). The combination of adenosine and L-Hcy (50 \textmu M), a condition we established to sensitize intracellular hypomethylation,\textsuperscript{6} did not change CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid} subset differentiation compared with adenosine control (Figure 7A and 7B). CAT, a hydrogen peroxide scavenger, together with SOD, a superoxide anion scavenger, reversed Hcy effects and brought the CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid} population to 11.6\% (P=0.002) of the controls with no L-Hcy. Similarly, the NAD(P)H oxidase inhibitor apocynin completely prevented Hcy-induced CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid} subset differentiation. In contrast, Hcy maintained the capacity to induce CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid} subset differentiation in the presence of the xanthine oxidase inhibitor allopurinol (Figure 7C), the peroxynitrite scavenger uric acid, and the nitric oxide synthase inhibitor L-NAME. SOD/CAT and apocynin slightly increased the baseline level of CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid}, whereas allopurinol, L-NAME, and uric acid did not. Interestingly, uric acid and L-NAME reduced CD11b\textsuperscript{+} Ly-6C\textsuperscript{mid} subset formation in the presence of Hcy, but not as much as SOD/CAT or apocynin.

**Discussion**

In the present study, we generated a novel genetic mouse model with severe HHcy and hypercholesterolemia, Tg-hCBS apoE\textsuperscript{−/−} Cbs\textsuperscript{−/−} mice, and examined the effects of HHcy on atherosclerotic lesion formation and monocyte accumulation. The Tg-hCBS apoE\textsuperscript{−/−} Cbs mouse described here is a useful model for HHcy-related atherosclerosis research. This model has a survival advantage over our previous apoE\textsuperscript{−/−} Cbs mice.\textsuperscript{17} The Tg-hCBS apoE\textsuperscript{−/−} Cbs mice retained severe HHcy and survived normally. The control Tg-hCBS apoE\textsuperscript{−/−} Cbs mice fed a HF diet have plasma Hcy levels of 13.9...
μmol/L, similar to what we have observed in the apoE−/− Cbs−/− mice fed the same diet (14.2 μmol/L).\textsuperscript{17} Plasma Hcy levels in the Tg hCBS apoE−/− Cbs−/− mice on regular diet is 200 μmol/L, which is higher than 169 μmol/L in the Tg hCBS Cbs−/− mice.\textsuperscript{24} These data support the notion that hyperlipidemia increases Hcy levels.

The Tg-hCBS apoE−/− Cbs−/− mice were slightly smaller than their littermate controls. This was also found in Tg-S466L Cbs−/− mice (data not shown). These results are in accordance with our previous reports.\textsuperscript{24} We have observed a trend in decreasing spleen weight in severe HHcy Tg-hCBS apoE−/− Cbs−/− mice (Figure 1G), suggesting that HHcy causes growth retardation in general and reduces spleen weight, potentially creating a negative impact on immune system development.

We previously reported that HHcy accelerates atherosclerosis and increases LDL uptake by peritoneal macrophages in apoE−/− Cbs−/− and apoE−/− Cbs−/− mice with and without dietary perturbation.\textsuperscript{17} The present study is in agreement with our previous findings and provides further evidence demonstrating that severe HHcy accelerates atherosclerosis and increases Ly-6C−positive monocyte/macrophage inflammatory subset accumulation in the atherosclerotic lesion (Figure 2). These findings led to the new hypothesis that severe HHcy contributes to atherosclerosis via promoting inflammatory monocytes.
It has been suggested that inflammatory processes play a pivotal role throughout atherosclerotic progression. We found that plasma TNF-α and MCP-1 levels were significantly elevated in Tg-hCBS apoE−/− Cbs−/− mice and were positively correlated with plasma Hcy levels (Figure 3). TNF-α is a proinflammatory cytokine that activates the endothelium. MCP-1 is a chemokine that mediates monocyte adhesion and infiltration into inflammatory sites. It was suggested that Hcy-induced MCP-1 expression in aortic endothelial cells may also contribute to leukocyte recruitment. Our findings support the hypothesis that HHcy promotes systemic inflammation and monocyte infiltration into the vessel wall.

Clinical criteria usually consider >8% circulating MNCs, based on their mononuclear morphological feature, as monocytes in humans. The control Tg-S466L Cbs mice animals have 3% to 5% MNCs (gate i) among the peripheral nucleated cells (gate I; Figure 4A). Severe HHcy induced monocytosis (8.6% MNCs) at 8 months of age. Monocytosis was also observed in the control Tg-hCBS apoE−/− Cbs−/− mice (10.3% MNCs) and was exacerbated by HHcy in Tg-hCBS apoE−/− Cbs−/− mice (11.6% MNCs; Figure 4C). Using CD11b as a monocyte molecular marker, we found that severe HHcy significantly promoted monocyte expansion in peripheral blood, spleen, and BM in both mouse lines independently of hyperlipidemia (Figure 4B and 4D).

Recent studies have shown that circulating monocytes display heterogeneity, which commits to specific functions in atherogenesis. Using Ly-6C as a marker, we can divide...
monocytes into 3 subsets: Ly-6C\textsuperscript{hi}, Ly-6C\textsuperscript{mid}, and Ly-6C\textsuperscript{low} fractions.\textsuperscript{23,27} The Ly-6C\textsuperscript{hi} monocytes are recognized as the inflammatory subset, which can differentiate into macrophages in atheromas of apoE\textsuperscript{-/-} mice.\textsuperscript{13} The Ly-6C\textsuperscript{mid} monocytes are considered the intermediate inflammatory subset because of their ability to uptake inflammatory particles and to migrate to sites of inflammation.\textsuperscript{27} We found that severe HHcy was associated with increases in both Ly-6C\textsuperscript{hi} and Ly-6C\textsuperscript{mid} monocyte subsets in peripheral blood, spleen, and BM in both mouse models, which were further increased with age (Figure 5). Therefore, we have demonstrated for the first time that HHcy selectively promotes the expansion of inflammatory monocyte subsets in the periphery and BM in mice independently of hyperlipidemia.

Our data indicate that mouse primary splenocytes are a relevant in vitro model of monocyte survival and differentiation.\textsuperscript{13} The mouse spleen, a hematopoietic organ, was used to study monocyte/macrophage heterogeneity and in vitro monocyte differentiation.\textsuperscript{13,14} Here, we have demonstrated an Hcy-specific effect on promoting Ly-6C monocyte differen-
tiation that is not mimicked by Cys. The distinct effects of Hcy and Cys on monocyte differentiation may be related to the different biochemical activities of these amino acids. Our study is the first to demonstrate that HHcy induces monocyte expansion and selectively promotes differentiation of Ly-6Chi and Ly-6Cmid inflammatory monocytes in the diseased models of severe HHcy. The modulation of HHcy on monocyte heterogeneity may enhance inflammatory responses during atherogenesis and contribute to the increased risk of CVD in HHcy.

Finally, we demonstrated that Hcy-induced inflammatory monocyte differentiation was prevented by SOD/CAT and the NAD(P)H oxidase inhibitor apocynin and partially reduced by uric acid and L-NAME. These data suggest that oxidant stress, especially superoxide anion, resulting mostly from NAD(P)H oxidase activation, is the major mechanism mediating Hcy-induced Ly-6Cmid inflammatory monocyte differentiation. Because Hcy did not induce the Ly-6Cmid subset in the presence of adenosine, a condition we established to sensitize intracellular hypomethylation,6 we ruled out a hypomethylation mechanism. It has been reported that Hcy induced MCP-1 secretion in human monocytes via oxidant stress.28 Moreover, Hcy-stimulated superoxide anion production is mediated by NAD(P)H oxidase activation via protein kinase Cβ in human monocytic cell line THP-1.29

Future studies examining NAD(P)H oxidase regulation should provide important insight into Hcy-induced monocyte differentiation and atherosclerosis.

Conclusions

Our studies provide the first evidence demonstrating that severe HHcy induces systematic inflammation and accelerates atherosclerosis in a novel model of severe HHcy and hypercholesterolemia. We found that HHcy induces inflammatory monocyte subset differentiation in mice independently of hyperlipidemia and in cultured splenocytes mostly via NAD(P)H oxidase–mediated oxidant stress. Our studies indicate that HHcy-induced inflammatory monocyte subset differentiation may be responsible for the increased risk of CVD in HHcy.

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Disclosures

None.

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Hyperhomocysteinemia decreases circulating high-density lipoprotein by inhibiting apolipoprotein A-I protein synthesis and enhancing HDL cholesterol clearance.


**CLINICAL PERSPECTIVE**

Hyperhomocysteinemia (HHcy) is an independent risk factor for cardiovascular diseases. In this study, we established novel disease models with severe HHcy and hypercholesterolemia in which the mouse cystathionine β-synthase (CBS) and apolipoprotein E (apoE) genes are deficient and an inducible human CBS or disease-relevant mutant CBS (S466L) transgene is introduced to circumvent the neonatal lethality of CBS deficiency (Tg-hCBS apoE<sup>−/−</sup> Cbs<sup>−/−</sup> or Tg-S466L Cbs<sup>−/−</sup> mice). First, we demonstrated that severe HHcy accelerated atherosclerosis and inflammatory monocyte/macrophage accumulation in the lesion and increased plasma tumor necrosis factor-α and monocyte chemotactrant protein-1 levels in Tg-hCBS apoE<sup>−/−</sup> Cbs<sup>−/−</sup> mice fed a high-fat diet. Thus, systemic inflammation possibly mediated the enhanced Ly-6C<sup>+</sup> monocyte/macrophage accumulation in the lesion. We also found that severe HHcy induced Ly-6C<sup>+</sup> and Ly-6C<sup>+</sup> inflammatory monocyte subset expansion in the peripheral blood, spleen, and bone marrow in Tg-hCBS apoE<sup>−/−</sup> Cbs<sup>−/−</sup> and Tg-S466L Cbs<sup>−/−</sup> mice independently of hyperlipidemia. Furthermore, we discovered that l-homocysteine, but not l-cysteine, promoted Ly-6C<sup>+</sup> monocyte subset survival and Ly-6C<sup>+</sup> subset differentiation in cultured primary mouse splenocytes. Finally, we reported that Hcy-induced Ly-6C<sup>+</sup> subset differentiation was abolished by superoxide dismutase/catalase or the NAD(P)H oxidase inhibitor apocynin. Taken together, these results show that HHcy selectively promoted Ly-6C<sup>+</sup> monocyte subset differentiation and enhanced their accumulations in the lesion, contributing to accelerated atherosclerosis in HHcy. Thus, the present study provides important insights into HHcy-related cardiovascular disease.
Hyperhomocysteinemia Promotes Inflammatory Monocyte Generation and Accelerates Atherosclerosis in Transgenic Cystathionine β-Synthase–Deficient Mice
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Supplemental Material

Supplemental Methods:

**Body and organ weight measurements** — At the end of each experiment, mouse body weights were recorded. Heart, liver, kidney and spleen samples were collected and weighed to evaluate organ development. In addition, tibia length was measured. Organ weight is expressed as a proportion of tibia bone length (mg/mm) to reduce variation due to overall body weight.

**Hcy and methionine (Met) measurements** — Plasma from 4-hour fasted mice was collected into a 4 mM EDTA-coated tubes at the end of each experiment. A total of 50 µL plasma was then analyzed for Hcy and Met levels using a Biochrom 30 amino acid analyzer (Cambridge, UK) as previously described.¹

**Aortic sinus cross-sectioning and lesion analysis** — Tg-hCBS apoE⁻/⁻ Cbs⁻/⁻ mice and their controls were sacrificed at the age of 16 weeks after 8 weeks on the HF diet. Hearts were harvested, weighed, stored in OCT compound (Tissue Tek; Sakura Finetek USA, Torrance, CA), and quick-frozen on dry ice. Serial cross sections of the aortic root were prepared as previously described² with modification. Cryostat sections (10µm) were taken from where the 3 aortic valves first appeared up to where the aortic valves disappeared, and mounted on Superfrost Excell microscope slides (Fisher Scientific, Pittsburgh, PA). About 64 sections were collected and divided over 8 slides, so that every slide contained 8 sections covering 640 µm intervals. Slides were stained with oil red O and counterstained with hematoxylin for lesion evaluation. For
monocyte/macrophage identification, the slides were first blocked with 5% donkey serum for 30min, then sequentially incubated with a rat anti-mouse MOMA-2 monoclonal antibody (Chemicon, Ramona, CA), a donkey anti-rat IgG-fluorescein isothiocyanate (FITC) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), then with a mouse anti-mouse Ly-6C monoclonal antibody (Abcam, Cambridge, MA) and a donkey anti-mouse IgG-rhodamine red-X (Rdx) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), and finally with 4', 6-diamidino-2-phenylindole (DAPI) for nuclear staining. Normal rat IgG2b was used as the isotype control.

Atherosclerosis lesion area was defined as the neointimal region between the lumen and internal elastic lamina (IEL). The percent lesion area of the aortic sinus was calculated by dividing lesion area by the sinus area, which is circumscribed by the IEL. The average value of 8 sections on each slide was measured by a single observer blinded to the experimental protocol and used for analysis. The monocyte area in the lesion was determined by MOMA-2–positive staining.

**Plasma tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 analysis** — Mouse plasma TNF-α and MCP-1 concentrations were determined using enzyme linked immunosorbent assay (ELISAs) kits from BD Biosciences (San Jose, CA) for TNF-α, and from Thermo Fisher Scientific (Rockford, IL) for MCP-1, respectively, according to the manufacturer’s
instructions. The lower limits of detection were 4 pg/mL for MCP-1, and 5 pg/mL for TNF-α.

**Peripheral blood, spleen, and bone marrow cell isolation** — Cells were isolated from mouse peripheral blood, spleen and bone marrow. Bone marrow cells from both tibias and femurs were harvested by inserting needles into the bone and washing with HBSS (Cellgro; Mediatech Inc.) supplemented with 2% (v/v) FCS and filtered through 70 μM cell strainer (BD Falcon, San Jose, CA). Peripheral blood was drawn via postorbital puncture with heparinized micro-hematocrit capillary tubes (BD, Franklin Lakes, NJ). Spleens were removed, homogenized gently between the frosted ends of the slides at 4°C, and filtered through a 70μM cell strainer (BD Falcon, San Jose, CA). Red blood cells in these three tissues were lysed with ACK lysis buffer. The resulting single-cell suspensions were washed with HBSS supplemented with 2% (v/v) FCS and 0.09% (w/v) NaN₃ for flow cytometric analysis, or washed with designed cell culture medium for the in vitro studies.

**Flow cytometry analysis** — Isolated peripheral blood, spleen and bone marrow (BM) cells were co-incubated with monoclonal antibodies to CD11b (anti-CD11b, clone M1/70)–PE and Ly-6C (anti-Ly6C, AL21)-FITC (BD Pharmingen™, San Diego, CA). Flow cytometry analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA). Data were analyzed using the FlowJo software (Tree Star Inc., Ashland, OR). First, nucleated cells were identified as the gate i after excluding red blood cells. Mononuclear cells (MNC) were selected in the gate ii by distinguishing MNC from granulocytes and lymphocytes with their lower
granular content, as reflected in lower side-scatter light (SSC), and their larger cell size, as reflected in higher forward scatter light (FSC). Finally, monocytes were identified from the gate ii MNC population based on their high level expression of CD11b. Therefore, monocytes were defined as CD11b+ MNC, which were further divided into three subgroups based on their Ly-6C expression levels (low, middle and high).

**Statistical Analysis** — Each experiment was repeated at least 3 times. Statistical analyses were performed with SigmaStat 2.03 (SPSS Science, Chicago, IL). Results are expressed as the mean ± SD or SEM. Statistical comparison of single parameters between two groups was performed by independent t test. Correlations between plasma Hcy concentration and the variables were performed using Spearman correlation analysis. A probability value p <0.05 was considered to be significant.
Supplemental Reference:

