Increased Monocyte Adhesion to Aortic Endothelium in Rats With Hyperhomocysteinemia
Role of Chemokine and Adhesion Molecules

Guoping Wang,* Connie W.H. Woo,* Fion L. Sung, Yaw L. Siow, Karmin O

Objective—The stimulatory effect of homocysteine (Hcy) on monocyte chemoattractant protein (MCP)-1 expression in vitro has been suggested to play an important role in Hcy-mediated atherosclerosis. We investigated whether such a stimulatory effect occurs in vivo, leading to monocyte adhesion to the endothelium.

Methods and Results—Sprague-Dawley rats were divided into 4 groups. Hyperhomocysteinemia was induced in 1 group of rats after 4 weeks of a high-methionine diet (serum Hcy levels were 4- to 5-fold higher than levels in control rats). The number of ED-1–positive cells present on the surface of aortic endothelium was significantly elevated in hyperhomocysteinemic rats. There was a significant increase in the expression of MCP-1, vascular cell adhesion molecule-1 (VCAM-1), and E-selectin in the endothelium. Antibodies recognizing MCP-1, VCAM-1, or E-selectin could abolish the enhanced monocyte binding to the aortic endothelium of hyperhomocysteinemic rats. Endothelium-dependent aortic relaxation was impaired in hyperhomocysteinemic rats.

Conclusions—These results suggest that in the absence of other known risk factors, hyperhomocysteinemia stimulates the expression of MCP-1, VCAM-1, and E-selectin in vivo, leading to increased monocyte adhesion to the aortic endothelium. Such an effect may contribute significantly to the development of atherosclerosis by facilitating monocyte/macrophage infiltration into the arterial wall. (Arterioscler Thromb Vasc Biol. 2002;22:1777-1783.)

Key Words: hyperhomocysteinemia ▪ atherosclerosis ▪ monocyte chemoattractant protein-1 ▪ cytokines ▪ monocytes

Hyperhomocysteinemia is now regarded as one of the important risk factors for cardiovascular and cerebral vascular disorders. Elevated homocysteine (Hcy) levels in the blood have been observed in a significant proportion of patients with coronary artery disease. Several plausible mechanisms for Hcy-induced atherosclerosis have been proposed. These include endothelial dysfunction, increased proliferation of smooth muscle cells, enhanced coagulability, and increased cholesterol synthesis in hepatocytes. Endothelial dysfunction is considered to be one of the important mechanisms contributing to atherogenesis. It has been proposed that Hcy-caused endothelial injury may be due to oxidative stress, attenuation of NO-mediated vasodilatation, and disturbance in the antithrombotic activities of the endothelium. On injury, endothelial cells are capable of producing various cytokines and growth factors that participate in inflammatory reactions in the arterial wall.

Dysfunction of endothelial cells is the key process promoting inflammatory reactions. One of the earliest detectable cellular responses in the formation of atherosclerotic lesions is the local recruitment of monocytes by the vascular endothe-
dependent vasodilation function, likely due to diminished NO bioactivity, was observed in the CBS-deficient mice (which had impaired Hcy metabolism). These studies indicate that Hcy may enhance vascular inflammation and endothelial dysfunction in animals that are prone to the development of atherosclerosis. However, it remains to be investigated whether hyperhomocysteinemia itself can stimulate endothelial production of chemokines and adhesion molecules.

Although results from in vitro studies suggest that Hcy, at pathophysiological concentrations, stimulates chemokine expression in vascular cells, it is unknown whether hyperhomocysteinemia can initiate similar changes, leading to enhanced monocyte adhesion/binding to the vascular endothelium in vivo. The objective of the present study was to investigate the in vivo effects of Hcy on monocyte adhesion/binding to vascular endothelium in rats with hyperhomocysteinemia and to elucidate the underlying mechanisms associated with this event.

Methods

Dietary Induction of Hyperhomocysteinemia in an Animal Model

Male Sprague-Dawley rats (bred from stock supplied by Charles River Laboratories, Wilmington, Mass) aged 8 weeks were divided into 4 groups (n=35 for each group) and maintained for 4 weeks on the following diets before the experiments: (1) control diet (regular diet), consisting of PMI Rodent Diet 5001 (PMI Nutrition International) containing methionine (0.49% [wt/wt]), folate (0.0066% [wt/wt]), and cysteine (0.34% [wt/wt]); (2) high-methionine diet, consisting of regular diet plus 1.7% methionine (wt/wt); (3) high-methionine plus folate-rich diet, consisting of regular diet plus methionine (1.7% [wt/wt]) and folate (0.006% [wt/wt]); and (4) high-cysteine diet, consisting of regular diet plus 1.2% cysteine (wt/wt). Results from our laboratory and others16 have suggested that a high-methionine diet for 4 weeks is sufficient to induce hyperhomocysteinemia. In the present study, a group of rats fed the high-methionine plus folate-rich diet was included to test whether lowering blood Hcy levels by folate supplement could prevent hyperhomocysteinemia-induced chemokine and/or adhesion molecule expression. In addition, a high-cysteine diet group was included to test the specificity of Hcy effect on chemokine expression in the animal model. Cysteine is another thiol-containing amino acid and has been shown to have no effect on the expression of MCP-1 and its receptor (CCR2) in vitro.12 The serum Hcy levels were measured with the IMx Hcy assay (Abbott Diagnostics Division), which is based on fluorescence polarization immunoassay technology, and the folate levels were measured with IMx folate assay (Abbott Diagnostics Division), which is based on ion-capture technology.

Assessment of Endothelial Function

The thoracic aorta was isolated, cut longitudinally, and mounted on a glass slide with the endothelial side up. Mouse monoclonal antibodies against rat ED-1 on the surface of monocytes/macrophages (1:100, Serotec) were added, and the incubation was carried out for 30 minutes. The secondary antibodies for immunostaining were fluorescein-conjugated (FITC-labeled) goat anti-mouse immunoglobulin antibodies (Calbiochem-Novabiochem Corp). Monocytes/macrophages bound to the aortic endothelium were identified with the use of a fluorescence microscope (Zeiss Axiosplan2 Universal Microscope). The number of monocytes/macrophages bound to the endothelium was counted from 9 equally distributed sites on each aortic segment.

Detection of Monocyte Binding to Aortic Endothelium

In the in vitro binding of monocytes to the aortic endothelium isolated from rats was examined by monocyte binding assay.19 First, THP-1 mononuclear cells (TIB-202, American Type Culture Collection) were labeled with fluorescein by incubation in RPMI 1640 medium containing 3 μg/mL tetramethylrhodamine isothiocyanate (Calbiochem). Second, the thoracic aorta (30 mm) was isolated from rats and opened longitudinally. After incubation of the aortic segments with fluorescein-labeled monocytes (5×10⁵ cells/mL) for 30 minutes, the adherent monocytes were counted by fluorescence microscopy from 9 equally distributed sites on each segment. For the antibody-blocking test, the thoracic segments were incubated with respective antibodies 1 hour before incubation with fluorescein-labeled monocytes.

Immunohistochemistry

To detect the endothelial expression of MCP-1 and adhesion molecules, the thoracic aorta was isolated and divided into upper thoracic, middle thoracic, and lower thoracic segments. These segments were immersion-fixed in 10% neutral-buffered formalin overnight and then embedded in paraffin. Sequential 5-μm paraffin-embedded cross sections were prepared. Immunohistochemical analysis was performed to detect MCP-1, vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, E-selectin, and P-selectin. For detection of MCP-1, rabbit polyclonal antibodies (1:100, Pepro Tech EC Ltd) against rat MCP-1 were used. Endogenous peroxidase was blocked with 0.3% H₂O₂ for 20 minutes. The secondary antibodies for immunostaining were biotin-conjugated anti-rabbit immunoglobulins (1:200, Dako). For the detection of adhesion molecules in the aorta, goat polyclonal antibodies (1:100) against rat VCAM-1, ICAM-1, E-selectin, and P-selectin were used as primary antibodies, respectively (Santa Cruz Biotechnology). The secondary antibodies were biotin-conjugated rabbit anti-goat immunoglobulins (1:250, Dako).

Statistical Analysis

The results were analyzed by using the 2-tailed independent Student t test. The level of statistical significance was set at P<0.05.

Results

Dietary Induction of Hyperhomocysteinemia

Hyperhomocysteinemia was induced in rats by feeding them a high-methionine diet. The high-methionine diet resulted in a 4- to 5-fold increase in the plasma Hcy levels (24.2±4.0 versus 5.3±0.8 μmol/L in control rats). Serum Hcy levels were significantly lower in rats fed the high-methionine plus folate-rich diet than in rats fed the high-methionine diet (8.6±1.5 versus 24.2±4.0 μmol/L, respectively). There was no significant elevation of serum Hcy levels in rats fed the high-cysteine diet (6.9±0.6 versus 5.3±0.5 μmol/L in control rats). These results indicated that rats fed the high-methionine diet for 4 weeks developed hyperhomocysteinemia. The folate level was significantly elevated in rats fed the high-methionine plus folate-rich diet (171.2±12.9 versus
83.5 ± 10.5 ng/mL in the control rats). However, there were no significant differences in serum folate levels among the control, high-methionine–fed, and high-cysteine–fed groups. Furthermore, there were no significant differences in body weights among rats fed different diets.

Effect of Hyperhomocysteinemia on Endothelial Function of the Aorta

The endothelial function of the aorta was determined by measuring the vascular response to ACh and SNP. Endothelium-dependent relaxation to ACh was significantly reduced in the aortas of hyperhomocysteinemic (high methionine–fed) rats compared with control rats (Figure 1A). On the other hand, endothelium-independent relaxation to SNP did not significantly differ between the control and hyperhomocysteinemic rats (Figure 1B). In addition, endothelium-dependent relaxation and endothelium-independent relaxation did not differ between the control and high-methionine plus folate–fed rats (data not shown).

Enhanced Monocyte/Macrophage Adhesion to Aortic Endothelium In Vivo

To determine whether the number of monocytes/macrophages present in the aortic endothelium was increased in hyperhomocysteinemic rats, en face immunofluorescence staining was performed with antibodies against ED-1 in freshly isolated aortic segments. Occasionally, cells positively stained with antibodies recognizing ED-1 were observed on the surface of the aortic endothelium isolated from rats fed the regular diet (Figure 2a). The number of ED-1–positive cells present in the endothelium of the aortas isolated from hyperhomocysteinemic rats was significantly higher than the number present in the control rats (Figure 2b), indicating an increase in monocytes/macrophages in the aortic endothelium in hyperhomocysteinemic rats. The number of ED-1–positive cells bound to the aortic endothelium was significantly reduced in rats fed the high-methionine plus folate-rich diet (Figure 2c) compared with rats fed the high-methionine diet. The number of ED-1–positive cells bound to the aortic endothelium of the rats fed the high-cysteine diet was similar to that of the control rats (Figure 2d). The nonspecific IgG did not result in positive staining in the aortic segment (Figure 2e). No atherosclerotic lesion was found in the aortic endothelium of rats fed the high-methionine diet.

Figure 1. Assessment of vessel relaxation. A, Endothelium-dependent relaxation response to cumulative doses of ACh in rat aortic segments isolated from the control group (filled squares) and high-methionine fed group (filled circles) was examined. B, Endothelium-independent relaxation response to cumulative doses of SNP in rat aortic segments isolated from control and high-methionine–fed group was examined. Each point represents mean ± SEM. *P < 0.05 compared with values obtained from control group.

Figure 2. En face immunofluorescence staining of monocytes bound to the endothelium of rat aorta. Thoracic aortas were isolated from rats fed the regular diet (control, a), high-methionine diet (Met, b), high-methionine plus folate-rich diet (Met + folate, c), and high-cysteine diet (cysteine, d). ED-1–positive cells were identified by fluorescence microscopy at a magnification of ×200. Nonspecific IgG was used as a negative control (e). Photomicrographs are representative of 5 separate experiments. Arrowheads point to ED-1–positive cells. Results are expressed as mean ± SD (error bar). *P < 0.05 compared with control values; #P < 0.05 compared with values obtained from rats fed the high-methionine diet.
Enhanced Binding of Monocytes to Aortic Endothelium In Vitro

Next, experiments were performed to determine whether there was any difference in the capacity of monocyte binding to the aortic endothelium isolated from rats fed different diets in vitro. There were a few fluorescein-labeled monocytes bound to the aortic endothelium isolated from rats fed the regular diet (please see online Figure Ia, which can be accessed at http://atvb.ahajournals.org). There were more monocytes bound to the aortic endothelium of hyperhomocysteinemic rats compared with control rats (292% versus 100%, respectively; please see online Figure Ib). These results suggest that the binding of monocytes to the aortic endothelium was significantly increased in hyperhomocysteinemic rats. The number of monocytes bound to the aortic endothelium of aortas isolated from rats fed the high-methionine plus folate-rich diet was significantly lower than that from rats fed the high-methionine diet (135% versus 292% and 107% versus 292%, respectively; please see online Figure Ic and Id).

Effect of Hyperhomocysteinemia on Expression of MCP-1 and Adhesion Molecules in Aortic Endothelium

The immunostaining for MCP-1 protein was minimal in the upper, middle, and lower thoracic segments of the aortas isolated from rats fed the regular diet (Figure 3). In contrast, the immunostaining for MCP-1 was stronger in the endothelium of all aortic segments prepared from rats fed the high-methionine diet (Figure 3), indicating an elevation of MCP-1 expression in the aortic endothelium of hyperhomocysteinemic rats. On the other hand, the expression of MCP-1 in rats fed the high-methionine plus folate-rich diet or fed the high-cysteine diet was similar to that in rats fed the regular diet (Figure 3).

Because increased monocyte binding to the aortic endothelium might also be influenced by the presence of adhesion molecules, the expression of VCAM-1, ICAM-1, E-selectin, and P-selectin was analyzed. There was a marked increase in immunostaining for VCAM-1 (Figure 4A) and E-selectin (Figure 4B) in the endothelium of the aortas isolated from rats fed the high-methionine diet. The expression of VCAM-1 (Figure 4A) and E-selectin (Figure 4B) was significantly reduced in the aortic endothelium of rats fed the high-methionine plus folate-rich diet compared with rats fed the high-methionine diet. There was no change in immunostaining for ICAM-1 (Figure 4C) and P-selectin (Figure 4D) in rats fed the high-methionine diet compared with rats fed the regular diet.

To determine whether increased expression of MCP-1, VCAM-1, and E-selectin was responsible for enhanced monocyte binding to the aortic endothelium of hyperhomocysteinemic rats, the isolated aorta was incubated with antibodies recognizing MCP-1 or adhesion molecules before the monocyte-binding assay. Pretreatment with anti-MCP-1 antibodies, anti-VCAM-1 antibodies, or anti-E-selectin antibodies significantly reduced the numbers of monocytes binding to the aortic endothelium isolated from hyperhomocysteinemic rats (please see online Figure II, which can be
accessed at http://atvb.ahajournals.org). These results suggest that enhanced monocyte binding to the aortic endothelium in hyperhomocysteinemic rats is mediated, in part, by increasing the expression of MCP-1, VCAM-1, and E-selectin. On the other hand, treatment with antibodies against ICAM-1 or P-selectin or nonspecific IgG antibodies did not result in any change in the numbers of monocytes binding to the aortic endothelium isolated from hyperhomocysteinemic rats (please see online Figure II). The expression of ICAM-1 and P-selectin did not appear to contribute significantly to the enhanced monocyte binding to the aortic endothelium in these animals.

**Discussion**

In the present study, we have observed that diet-induced elevation of plasma Hcy levels can stimulate the expression of chemokine (MCP-1) and adhesion (VCAM-1 and E-selectin) molecules in the aortic endothelium. As a consequence, monocyte/macrophage adhesion to the aortic endothelium was significantly elevated. Endothelium-dependent relaxation of the aorta was impaired in hyperhomocysteinemic rats.

After 4 weeks of dietary treatment, plasma Hcy levels were significantly elevated in rats fed the high-methionine diet. On the other hand, there was a significant reduction in plasma Hcy levels in rats fed the high-methionine plus folate-rich diet. Although no visible atherosclerotic lesion was found in the aortic endothelium of hyperhomocysteinemic rats, a significant increase in the adhesion and binding of monocytes to the endothelium was observed. The adhesion of leukocytes, including monocytes, to arterial endothelium is a common feature linking the inflammation reaction and the development of early atherosclerosis.8–10 Increased monocyte/macrophage binding and adhesion to the vascular endo-

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Immunohistochemical staining for VCAM-1, E-selectin, ICAM-1, and P-selectin in the aortic endothelium. Cross sections of thoracic aortas were prepared from rats fed a regular diet (control), high-methionine diet (Met), high-methionine plus folate-rich diet (Met+folate), or high-cysteine diet (cysteine). Immunohistochemical staining for VCAM-1 (A), E-selectin (B), ICAM-1 (C), and P-selectin proteins (D) was performed with respective antibodies. VCAM-1, E-selectin, ICAM-1, and P-selectin proteins were identified by light microscopy at a magnification of ×400. Representative photos were obtained from 5 separate experiments. Arrowheads point to the endothelial cell monolayer.
thelium may represent an early feature of atherosclerotic development in hyperhomocysteinemia. Results obtained from the present study demonstrate, for the first time, that diet-induced chronic hyperhomocysteinemia stimulates the interaction between monocytes/macrophages and the aortic endothelium.

In the present study, the mechanisms underlying the enhanced monocyte adhesion were investigated. MCP-1 is a potent chemoattractant protein for monocytes. The expression of this chemokine has been shown to increase significantly during atherogenesis. Indeed, we observed that endothelial MCP-1 expression was significantly elevated in hyperhomocysteinemic rats. MCP-1 might initiate the binding of monocytes to the aortic endothelium via its strong chemotactic property. This was supported by our observation that antibodies recognizing MCP-1 could block the binding of monocytes to the endothelium of the aortas isolated from hyperhomocysteinemic rats. In addition, our results demonstrated that hyperhomocysteinemia caused a significant increase in the endothelial expression of VCAM-1 and E-selectin while not altering the expression of ICAM-1 and P-selectin. Inasmuch as pretreatment with antibodies against either VCAM-1 or E-selectin could markedly block monocyte binding to the aortic endothelium of rats fed the high-methionine diet, enhanced expression of these 2 adhesion molecules might contribute to hyperhomocysteinemia-induced monocyte adhesion to the vascular endothelium. It has recently been reported that the expression of VCAM-1 increases in apoE-null mice with hyperhomocysteinemia. VCAM-1 and E-selectin are inducible adhesion molecules expressed by endothelial cells. Together with MCP-1, these 2 adhesion molecules bind to circulating monocytes, leading to cell attachment and migration into the subendothelial space. Our results indicate that increased expression of MCP-1, VCAM-1, and E-selectin might be responsible for enhanced monocyte adhesion/binding to the vascular endothelium in hyperhomocysteinemia. Although the expression of ICAM-1 and P-selectin has been shown to be upregulated in atherosclerotic lesions, we did not observe a significant increase in the expression of these 2 adhesion molecules in the aortic endothelium of hyperhomocysteinemic rats. Treatment of the endothelium with antibodies specific for ICAM-1 and P-selectin could not block the increased monocyte binding to the endothelium of hyperhomocysteinemic rats. These results further support the notion that these 2 adhesion molecules might not contribute significantly to the enhanced monocyte binding to the aortic endothelium induced by hyperhomocysteinemia. It is also possible that ICAM-1 and P-selectin might be involved in the later stages of atherogenesis, ie, after the stage set by MCP-1, VCAM-1, and E-selectin in hyperhomocysteinemic rats. It has recently been reported that mild hyperhomocysteinemia in mice due to heterozygous CBS gene deficiency leads to endothelial dysfunction and increased expression of P-selectin. The differential contributions of the individual adhesion molecules involved in monocyte binding in the pathogenesis of atherosclerosis in different species remain to be investigated in future studies.

Oral folate supplementation has been shown to improve the arterial endothelium-dependent vascular function of the brachial artery in healthy subjects with mild hyperhomocysteinemia. In a recent study, administration of folate and vitamin B<sub>12</sub> for 9 weeks to patients with coronary heart disease and hyperhomocysteinemia was shown to improve vascular endothelial function as assessed by brachial artery flow-mediated dilatation. Although lowering plasma Hcy levels may contribute to the improved vascular function, folate has been shown to possess some independent antioxidative capacity. Furthermore, its ameliorative effect on endothelial NO synthase may produce more benefit in the early stages of atherogenesis. In the present study, we observed that folic supplementation to the rats fed the high-methionine diet prevented an elevation of Hcy levels in the blood. Such an Hcy-lowering effect might contribute to the inhibition of expression of MCP-1 and adhesion molecules as well as monocyte adhesion to endothelial cells. On the other hand, its beneficial effect on endothelial function, on the expression of MCP-1, and on adhesion molecules could be due to the combined effect of Hcy lowering and the antioxidative property of folate.

It has recently been reported that hyperhomocysteinemia in rats produced by folate depletion impairs endothelium-dependent relaxation of coronary microvessels and carotid arteries and is accompanied by increased arterial permeability and arterial stiffening. It has recently been reported that hyperhomocysteinemia impaired the endothelium-dependent relaxation of the aorta although it did not alter the endothelium-independent vessel relaxation. This might be due to oxidative stress and the attenuation of bioactive NO, leading to impaired vasodilatation. The findings of the present study not only complement those observations but also indicate that hyperhomocysteinemia can act independently in the development of vascular dysfunction by upregulating the expression of MCP-1 and some adhesion molecules in the vascular endothelium. Furthermore, our results also indicate that hyperhomocysteinemia is associated with reduced endothelium-dependent vessel relaxation, indicating endothelial dysfunction in dietary-induced hyperhomocysteinemia.

To the best of our knowledge, this is the first study to show that in the absence of other known risk factors, dietary-induced hyperhomocysteinemia enhances the adhesion of circulating monocytes/macrophages to the aortic endothelium. Such a stimulatory effect is the result of increased expression of inflammatory markers, including MCP-1, VCAM-1, and E-selectin, in vascular endothelial cells. Elevation of monocyte adhesion to the vascular wall together with impaired endothelium-dependent relaxation may contribute to the development of atherosclerosis in hyperhomocysteinemia.

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Figure I. *In vitro* binding of monocytes to the endothelium of rat aorta. The thoracic aortas were isolated from rats fed with (a) regular diet (Control), (b) high-methionine diet (Met), (c) high-methionine plus folate-rich diet (Met+Folate) and (d) high-cysteine diet (Cysteine). Fluorescein-labeled monocytes bound to the endothelium were identified under a fluorescence microscope at a magnification of 200X. Photomicrographs are representatives from five separate experiments. Arrowheads point to fluorescein-labeled monocytes. Results are expressed as mean ± SD (error bar). *P* < 0.05 when compared with control values and #*P* < 0.05 when compared with values obtained from rats fed with a high-methionine diet.
**Figure II.** *In vitro* binding of monocytes to the endothelium of rat aorta in the presence of antibodies.

The thoracic aortas were isolated from rats fed with regular diet (Control) and high-methionine diet (Met). The aortas were cut longitudinally and the binding assay was performed after the following treatment: aortic segment isolated from the control rat (a), aortic segment isolated from high-methionine fed rat (b), aortic segment isolated from high-methionine fed rat pre-treated with (c) anti-MCP-1 antibodies (MCP-1 Ab), (d) anti-E-selectin antibodies, (e) anti-VCAM-1 antibodies, (f) anti-P-selectin antibodies, (g) anti-ICAM-1 antibodies and (h) non-specific IgG. Fluorescein-labeled monocytes bound to the endothelium were identified under fluorescence microscope at a magnification of 200X. Photomicrographs are the representatives from five separate experiments. Results are expressed as mean ± SD (error bar). 

*P< 0.05 when compared with control values, #P< 0.05 when compared with values obtained from rats fed with a high-methionine diet.