Shear Stress Inhibits Homocysteine-Induced Stromal Cell–Derived Factor-1 Expression in Endothelial Cells

Mao-Lin Sung, Chia-Ching Wu, Hsin-I Chang, Chia-Kuang Yen, Heng Jung Chen, Ju-Chien Cheng, Shu Chien, Cheng-Nan Chen

Rationale: Hyperhomocysteinemia contributes to vascular dysfunction and risks of cardiovascular diseases. Stromal cell–derived factor (SDF)-1, a chemokine expressed by endothelial cells (ECs), is highly expressed in advanced atherosclerotic lesions. The interplays among homocysteine, chemokines, and shear stress in regulating vascular endothelial function are not clearly understood.

Objective: To investigate the mechanisms for modulations of EC SDF-1 expression by homocysteine and shear stress.

Methods and Results: Homocysteine stimulation induced dose- and time-dependent SDF-1 expression and phosphorylation of mitogen-activated protein kinases extracellular signal-regulated kinase, c-Jun N-terminal kinase (JNK), and p38. By using specific inhibitors, small interfering (si)RNA, and dominant negative mutants, we demonstrated that activation of JNK pathway is critical for the homocysteine-induced SDF-1 expression. Transcription factor ELISA and chromatin immunoprecipitation assays showed that homocysteine increased Sp1- and AP-1–DNA binding activities in ECs. Inhibition of Sp1 and AP-1 activations by specific siRNA blocked the homocysteine-induced SDF-1 promoter activity and expression. Preshearing of ECs for 1 to 4 hours at 20 dyn/cm² inhibited the homocysteine-induced JNK phosphorylation, Sp1 and AP-1 activation, and SDF-1 expression. The homocysteine-induced SDF-1 expression was suppressed by NO donor. Inhibitor or siRNA for endothelial NO synthase abolished the shear inhibition of SDF-1 expression.

Conclusions: Our findings serve to elucidate the molecular mechanisms underlying the homocysteine induction of SDF-1 expression in ECs and the shear stress protection against this induction. (Circ Res. 2009;105:00-00.)

Key Words: homocysteine ■ endothelial cells ■ stromal cell–derived factor-1 ■ shear stress ■ transcriptional regulation

Vascular endothelial cells (ECs) are constantly exposed to fluid shear stress, the nature and magnitude of which play a significant role in the homeostasis of blood vessels. The preferential development of atherosclerosis at arterial branches and curvatures, where local flow is disturbed, suggests a role of shear stress in atherogenesis. Adhesion of circulating monocytes and T lymphocytes to ECs and their subsequent migration across the EC monolayer are early events in atherogenesis.1 Physiological levels of shear stress can modulate cellular signaling and EC function in ways that are protective against atherogenesis.2,3 A number of pathophysiologically relevant genes, such as adhesion molecules,4 growth factors,5 and chemokines,6 have been shown to be regulated by shear stress.

Chemokines play a significant role in atherosclerotic lesion development, primarily by inducing the transendothelial migration of leukocytes. Furthermore, chemokines may stimulate smooth muscle cell proliferation and migration from media to intima, as well as angiogenesis, thus influencing plaque formation, progression, and rupture.7 Chemokines are divided into CXC, CC, C, or CX3C based on their structural properties and primary amino acid sequence.8 Stromal cell–derived factor (SDF)-1, a member of the CXC family, is a strong chemoattractant in EPCs, lymphocytes, and monocytes9,10 and implicated in atherogenesis.11 SDF-1 has also been shown to induce platelet activation and aggregation via CXCR4 expressed on platelets, suggesting a link with atherothrombosis.12 SDF-1 expression can be regulated by extracellular factors derived from inflammatory or injured vascular tissues. The molecular mechanisms involved in the upregulation of
SDF-1 expression in pathophysiological conditions such as hyperhomocysteinemia is still unknown.

Homocysteine is a sulfur-containing amino acid formed during the metabolic demethylation of methionine. Elevated blood levels of homocysteine are related to higher risks of coronary heart disease, stroke, and peripheral vascular disease. Homocysteine may promote vascular damage and atherothrombosis via several mechanisms, including release of proinflammatory mediators, induction of endothelial dysfunction, and activation of apoptotic pathways in vascular cells. The relationship between the plasma levels of homocysteine and SDF-1, the mechanisms underlying homocysteine-induced SDF-1 expression, and the role of shear stress in the modulation of homocysteine-induced gene expression still remain unclear.

We aimed to establish the relationship between plasma homocysteine and SDF-1 levels in human blood and the interplay between shear stress and homocysteine stimulation in modulating endothelial gene expression by analyzing the effect of shear stress on homocysteine-induced SDF-1 expression in human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs). We found that the induction of SDF-1 expression by homocysteine was mediated via phosphorylation of c-Jun N-terminal kinase (JNK) and activation of the transcription factors (TFs) activator protein (AP)-1 and Sp1. Furthermore, fluid shear stress attenuated the homocysteine-induced SDF-1 expression at mRNA and protein levels, and ECs subjected to shear stress suppress the homocysteine-induced JNK phosphorylation and TF activation. In addition, homocysteine-induced SDF-1 expression was modulated by nitric oxide (NO): an NO donor suppressed homocysteine-induced SDF-1 expression, and inhibition of endothelial NO synthase (eNOS) attenuated shear stress inhibition of SDF-1 expression. These findings on the mechanisms of suppression of homocysteine-induced responses in ECs by shear stress provide new insights into the pathophysiology underlying the atheroresistance of straight segments of vascular tree.

Methods

Detailed information regarding materials and procedures for the following: subjects; measurement of plasma homocysteine; SDF-1 ELISA; EC culture; shear stress experiment; real-time quantitative polymerase chain reaction (PCR); Western blot analysis; reporter gene construct, small interfering (si)RNA, transfection, and luciferase assays; adenoviral infection; Sp1 and AP-1 TF assays (TF ELISAs); chromatin immunoprecipitation (ChIP) assay; and statistical analysis are provided in the Online Data Supplement at http://circres.ahajournals.org.

Results

Relationship Between Plasma Levels of Homocysteine and SDF-1

The mean plasma homocysteine concentration was significantly higher in high-risk patients (30.94 ± 4.13 μmol/L) than normal volunteers (14.08 ± 1.02 μmol/L). Linear regression shows a positive correlation between plasma homocysteine and SDF-1 levels in patients (Figure 1A; r = 0.81, P < 0.0001) and volunteers (Figure 1B; r = 0.51, P < 0.001). Linear regression of data combined the 2 groups was showed in Online Figure 1 (r = 0.79, P < 0.0001). We divided all subjects (patients plus normal volunteers) according to their plasma homocysteine levels into normal (<15 μmol/L, n = 24, 10 from patients), mild (16 to 30 μmol/L, n = 36, 11 from patients), and intermediate (31 to 100 μmol/L, n = 12, 11 from patients) hyperhomocysteinemia. Plasma SDF-1 level was significantly higher in subjects with intermediate hyperhomocysteinemia than normal or mild hyperhomocysteinemia (Figure 1C), indicating that higher levels of plasma homocysteine were associated with higher SDF-1.

Homocysteine-Induced SDF-1 Expression is Dose- and Time-Dependent

HUVECs and HAECs were stimulated with homocysteine at 100 μmol/L for the times indicated, or different doses (0 to 500 μmol/L) for 4 hours (for mRNA expression by real-time PCR) and 12 hours (for protein secretion by ELISA). In ECs, the SDF-1 mRNA level began to increase after 1 hour of homocysteine stimulation and reached highest level at 4 hours; thereafter, it gradually reduced to a level that is not significantly different from the static control in HUVECs at 12 hours but was still elevated at this time in HAECs (Figure 2A). SDF-1 protein in the conditioned medium also increased after stimulation (Figure 2C). The inductions of SDF-1 mRNA expression and protein secretion by homocysteine were dose-dependent (Figure 2B and 2D).

The viability of ECs was >94% when cultured for 24 hours with 20 to 200 μmol/L homocysteine and 87% with 500 μmol/L homocysteine (data not shown).

Homocysteine-Induced SDF-1 Expression in EC Was Mediated by JNK Pathway

Members of the mitogen-activated protein kinase superfamily are known to regulate EC gene expression and cellular
functions. The phosphorylation of extracellular signal-regulated kinase (ERK), JNK, and p38 in HUVECs increased rapidly after homocysteine stimulation, reaching maximal levels at 2 minutes for ERK and JNK, and 5 minutes for p38 (Figure 3A). After transient increases, phosphorylation decreased to nearly basal levels. (Online Figure II shows that JNK phosphorylation decreased to control at 60 minutes, but ERK and p38 phosphorylations were still elevated at 120 minutes.)

HUVECs were incubated with specific inhibitors for ERK (PD98059; 30 μmol/L), JNK (SP600125; 20 μmol/L), or p38 (SB203580; 10 μmol/L) for 1 hour before and during stimulation with homocysteine. The homocysteine-induced SDF-1 mRNA expression (Figure 3B) and protein secretion (Figure 3C) were significantly inhibited by SP600125, but not by PD98059 and SB203580. The homocysteine-induced SDF-1 mRNA expression was also inhibited by transfection with JNK-specific siRNA or infection with adenoviral dominant negative JNK (Ad-DN-JNK), but not by ERK- or p38-specific siRNA (100 μmol/mL each), nor with Ad-DN-ERK nor Ad-DN-p38 (Figure 3D). The effectiveness of these treatments was validated: ERK-, JNK-, and p38-specific siRNA (compared with control siRNA) caused a 75% reduction in ERK, JNK, and p38 protein expressions, respectively (data not shown). The DN-ERK, DN-JNK, and DN-p38 caused at least 80% inhibitions in homocysteine-induced ERK, JNK, and p38 phosphorylation (compared with control Ad-GFP) (data not shown).

AP-1– and Sp1-Binding Sites Are Essential for the Homocysteine Induction of Human SDF-1 Promoter Activity

The human SDF-1 gene promoter contains multiple TF binding sites, which have been shown to be responsive to different stimuli. To elucidate the cis-acting elements in the SDF-1 gene promoter that mediate the homocysteine-induced SDF-1 transcription, luciferase assay was conducted with p-1010-Luc plasmid and several deletion promoters constructs (Figure 4A). Transient transfection of ECs showed that the 5′-flanking region of human SDF-1 (−1010/+122) could drive transcription of a luciferase reporter (Figure 4B). Construction and analyses of 5′-deletion mutants in the −1010/+122 region of SDF-1, which directed maximum luciferase activity, showed that the activity decreased to ∼45% following sequence deletion from −430 to −214, to ∼75% after further deletion to −111, and was nearly abolished following 5′ deletion to −20 (Figure 4B).

To test whether AP-1 and Sp1 activations are involved in the signal transduction pathway leading to the homocysteine-induced SDF-1 gene expression, we transfected HUVECs with siRNA of Sp1 or c-jun, or incubated them with the specific inhibitor for Sp1 (mithramycin A, 100 nmol/L) for 1 hour, before stimulation with 50 or 100 μmol/L homocysteine for 4 hours. The homocysteine-induced SDF-1 mRNA expression (Figure 4C) and SDF-1 promoter activity (Figure 4D) were significantly reduced by inhibition with mithramycin A or siRNA of Sp1 or c-jun.

Homocysteine Induced Sp1– and AP-1–DNA Binding Activities

To investigate whether Sp1 and AP-1 bind the SDF-1 promoter region in HUVECs, we performed quantitative analysis for Sp1- and AP-1–binding activities in vitro by using TF ELISA kits (Panomics). Treatment of HUVECs with 100 μmol/L homocysteine caused both Sp1- and AP-1–DNA binding activities to increase at 30 minutes and remain elevated for at least 2 hours (Figure 5A and 5B, respectively). ChIP analysis was performed by subjecting immunoprecipitated chromosomal DNA with anti-Sp1 antibody to PCR using primers designed to amplify the SDF-1 promoter region harboring the Sp1-binding sites. Sp1 indeed bound to the SDF-1 promoter region containing the Sp1 sites (Figure 5C). Similarly, the DNA sequence including the AP-1 sites was specifically immunoprecipitated with anti-jun antibody (Figure 5D). These data suggested that the Sp1 and AP-1–binding sites play critical roles in the regulation of SDF-1 by homocysteine.

JNK Signaling Pathway Was Involved in Homocysteine-Induced SDF-1 Promoter Activity

To evaluate whether the inhibition of SDF-1 expression by JNK signaling pathway occurred at the transcriptional level,
we studied the effect of SP600125, JNK siRNA, and DN-JNK on homocysteine-induced SDF-1 promoter activity with the use of p1010-luc reporter construct that contains ~1010 bp of the proximal promoter sequences of human SDF-1 gene (p1010-Luc plasmid). Stimulation with homocysteine increases the luciferase activity significantly in HUVECs over the unstimulated control after normalization by transfection control (Figure 6A). Transfection of ECs with JNK siRNA or DN-JNK resulted in a marked inhibition of the homocysteine-induced SDF-1 promoter activity. However, transfection of cells with siRNA for ERK or p38, DN-ERK, and DN-p38 had little inhibitory effect on this homocysteine inducibility (Figure 6A). In HUVECs, transfection with siRNA or promoter constructs under the concentrations used does not cause cytotoxicity based on the cell number and morphology (data not shown).

To explore whether JNK activates the SDF-1 promoter leading to SDF-1 transcription via activation of Sp1 and AP-1, HUVECs were pretreatment with mitogen-activated protein kinase inhibitors, transfection with JNK siRNA, or infection with Ad-DN-JNK, followed by homocysteine stimulation, and then the Sp1 and AP-1 activations were assessed by TF ELISA kits. Homocysteine-induced AP-1 and Sp1-DNA binding activities were significantly inhibited in cells pretreatment with SP600125, JNK siRNA, or Ad-DN-JNK but not in pretreatment with PD98059, SB203580, control siRNA, or Ad-GFP (Figure 6B and 6C).

Preexposure of ECs to High Shear Stress Inhibited Homocysteine-Induced SDF-1 Expression
Preexposure of ECs to high shear stress (HSS) at 20 dyn/cm² for 4 hours significantly inhibited homocysteine-induced

Figure 2. Induction of SDF-1 expression in ECs by homocysteine stimulation. RNA samples were isolated at the indicated time periods or doses. All bar graphs represent folds of control ECs (CL) and normalized to 18S rRNA by real-time PCR analysis (A and B). The SDF-1 protein secretion was determined by ELISA analyses (A and D). ECs were stimulated with 100 μmol/L homocysteine for the times indicated (A and C) or stimulated with homocysteine at various doses for 4 hours (B) or 12 hours (D). Data are shown as means±SEM. *P<0.05 vs CL HUVECs; #P<0.05 vs CL HAECs.

Figure 3. JNK pathway is required for homocysteine-induced SDF-1 expression. A, HUVECs were kept as controls (CL) or stimulated with 100 μmol/L homocysteine, and the phosphorylations of ERK, JNK, and p38 were determined by Western blotting. B through D, HUVECs were kept as controls or stimulated with homocysteine for 4 hours (B and D) or 12 hours (C). Before being kept as controls or stimulated with homocysteine, ECs were (1) pretreated with PD98059 (PD), SP600125 (SP), or SB203580 (SB) individually for 1 hour (B and C); (2) transfected with control siRNA (si-CL) or a specific siRNA of si-ERK, si-JNK, or si-p38 (D); or (3) infected with control adenovirus (Ad-GFP), DN-ERK, DN-JNK, or DN-p38 (D). All bar graphs represent folds of control ECs and normalized to 18S rRNA (B and D). The SDF-1 secretion was determined by ELISA analyses (C). The results are shown as means±SEM. *P<0.05 vs CL; #P<0.05 vs vehicle control (DMSO in B and C), control siRNA (si-CL in D), or control adenovirus (Ad-GFP in D) with homocysteine stimulation.
SDF-1 mRNA expression (Figure 7A), and this effect is shear time–dependent in HUVECs (Online Figure III). Low shear stress (LSS) at 0.5 dyn/cm² did not have such an effect. Both HSS and LSS alone had no significant effect on SDF-1 expression (Figure 7A). In addition, HUVECs cultured on fibronectin or collagen type I and preexposed to HSS for 4 hours had similar inhibitory effect on homocysteine-induced SDF-1 expression (Online Figure IV). Preexposure of HUVECs to HSS resulted in a marked inhibition of the homocysteine-induced JNK phosphorylation (Figure 7B). Preexposure of HUVECs to HSS also resulted in a significant inhibition of the homocysteine-induced SDF-1 promoter activity (Online Figure V).

Preshearing of HUVECs at HSS for 4 hours significantly inhibited the homocysteine-induced Sp1- and AP-1–DNA binding activities in vivo by ChIP assays (Figure 7C and 7D) and in vitro by using TF ELISA kits (Online Figure VI, A and B). These results provide additional evidence that shear stress plays an important role in the inhibition of the JNK-, Sp1-, and AP-1–mediated SDF-1 expression in ECs induced by homocysteine stimulation.

**Figure 4.** The roles of Sp1 and AP-1 in homocysteine-induced SDF-1 mRNA expression and promoter activity. A, Human SDF-1 promoter constructs. The arrows indicate the TF binding sites. B, HUVECs were cotransfected with 5′-deletion constructs and stimulated with homocysteine for 4 hours. SDF-1 promoter activity was measured by luciferase assay normalized to β-galactosidase activity and shown relative to that of HUVECs transfected with p1010-Luc (set to 100%). SDF-1 mRNA (C) and SDF-1 p1010-Luc (D) activities were determined in HUVECs pretreated with vehicle (DMSO) or Sp1 inhibitor mithramycin A (MMA) or transfected with si-Cl, si-Sp1, or si-c-jun and then stimulated with homocysteine for 4 hours. All bar graphs represent folds of CL ECs; means±SEM. *P<0.05 vs CL; #P<0.05 vs vehicle control (DMSO) or si-CL with homocysteine stimulation.

**Figure 5.** Sp1- and AP-1– binding activities of the SDF-1 promoter region induced by 100 μmol/L homocysteine stimulation in HUVECs. Sp1 (A) activation and AP-1 (B) activation were determined by TF ELISA. All bar graphs represent folds of control HUVECs (CL); means±SEM. *P<0.05 vs CL. C and D, ChIP assay was performed for Sp1 by using Sp1 antibody (C) and AP-1 by using c-jun antibody (D).

**Figure 6.** JNK signaling pathway was involved in homocysteine-induced SDF-1 promoter activity. A, SDF-1 p1010-Luc activity was determined in HUVECs transfected with si-CL, si-ERK, si-JNK, or si-p38 or infected with Ad-GFP, DN-ERK, DN-JNK, or DN-p38 and then stimulated with homocysteine for 4 hours. Sp1 (B) and AP-1 (C) activities were determined by TF ELISAs in HUVECs pretreated with vehicle (DMSO), transfected with si-CL, si-JNK, or infected with Ad-GFP and DN-JNK and then stimulated with 100 μmol/L homocysteine for 2 hours. All bar graphs represent folds of control ECs; means±SEM. *P<0.05 vs CL; #P<0.05 vs vehicle control (DMSO), si-CL, or Ad-GFP with homocysteine stimulation.
Effect of NO on Homocysteine-Induced SDF-1 Expression

A major effect of hyperhomocysteinemia-induced EC dysfunction appears to be related to decreased bioavailability of EC-derived NO. To investigate whether the homocysteine-induced SDF-1 expression is modulated by NO, both HUVECs and HAECs were incubated with different doses of the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) for 4 hours before and during stimulation with homocysteine. The homocysteine-induced mRNA expression of EC SDF-1 was significantly inhibited by 20 to 100 μmol/L SNAP treatment (Figure 8A). Conversely, addition of 100 μmol/L N^G-nitro-L-arginine methyl ester (L-NAME) or transfection of eNOS siRNA before exposure to 4 hours of HSS abolished the shear-mediated inhibition of SDF-1 expression (Figure 8B). These results indicated that NO plays an important role in the homocysteine-induction and shear inhibition of SDF-1 expression in ECs.

Discussion

The production of proinflammatory factors in vascular cells play an important role in atherogenesis. SDF-1 is a potent chemokine that recruits mononuclear cells to the inflamed tissues and has been shown to be highly expressed in human atherosclerotic plaques. Moreover, increased levels of blood SDF-1 have been associated with stable coronary disease, and SDF-1 is likely to be involved in neointimal hyperplasia or thrombus formation after injury. The ability of homocysteine to stimulate SDF-1 gene expression in ECs may lead to the elevation of SDF-1 in the circulation during hyperhomocysteinemia. The mechanism by which homocysteine regulates SDF-1 gene expression of ECs, however, remains unclear. The novel findings of the present study are (Online Figure VII): (1) plasma homocysteine is positively correlated with SDF-1 in both high-risk patients and normal volunteers; (2) homocysteine stimulates SDF-1 mRNA expression and protein secretion in ECs; (3) homocysteine-

100 μmol/L L-NAME for 4 hours or transfected with si-CL or si-eNOS. Static ECs were kept with homocysteine without preshearing (static). *P<0.05 vs CL, MeOH-treated control (HSS+MeOH), or control siRNA (HSS+si-CL) HUVECs with homocysteine stimulation; #P<0.05 vs CL, vesicle control (HSS+MeOH), or control siRNA (HSS+si-CL) HAECs with homocysteine stimulation; &P<0.05 vs L-NAME–treated (HSS+L-NAME) or eNOS siRNA–transfected (HSS+si-eNOS) HUVECs with homocysteine stimulation; @P<0.05 vs MeOH-treated HAECs with homocysteine stimulation.
induced SDF-1 expression in ECs is mediated via JNK phosphorylation and Sp1 and AP-1 activation; (4) shear stress attenuates the homocysteine-induced SDF-1 expression; and (5) the effect of homocysteine and shear stress on EC SDF-1 expression is mediated by NO.

The results of this study demonstrate that homocysteine not only promotes the secretion of SDF-1 but also induces their gene transcription and expression in human ECs. Analysis of the human SDF-1 promoter activity with different plasmid constructs revealed that AP-1 and Sp1 function as the cis-element for homocysteine responsiveness via JNK phosphorylation. SDF-1 promoter has different binding sites for various transcriptional factors. Previous studies have shown that Sp1 and AP-1 can be activated through the JNK pathway in ECs. Regulation of gene expression through the use of combinations of different TFs such as Sp1 and AP-1 has been reported. In this study, we performed luciferase assays to show that Sp1 and AP-1 cooperate to activate the human SDF-1 promoter and we used TF ELISA and ChIP assays to demonstrate that the regulation of SDF-1 gene expression in ECs was mediated by increased Sp1- and AP-1–DNA binding activities following JNK phosphorylation. A previous study has shown that Sp1 induces a conformational change in the DNA that may contribute to the activation of AP-1 binding. The molecular details of Sp1 and AP-1 cooperation in activating the SDF-1 promoter need further investigation. Based on our results, we propose a possible signal transduction pathway in ECs in which homocysteine induces JNK phosphorylation, which activates Sp1 and AP-1, thus resulting in SDF-1 transcriptional activation.

Hyperhomocysteinemia has been defined as a plasma concentration of homocysteine exceeding 15 μmol/L and is considered severe at levels of >100 μmol/L. Severe hyperhomocysteinemia caused by genetic disorders usually involves homocysteine concentrations of >100 μmol/L. When untreated, affected individuals have a 50% chance of developing a major vascular disease. Mild hyperhomocysteinemia is quite prevalent in the general population (plasma homocysteine 15 to 50 μmol/L) but also has been shown to be associated with increased risks for cardiovascular diseases. Previous studies have demonstrated that homocysteine might induce the expression of chemokines, e.g., monocyte chemoattractant protein (MCP)-1 and interleukin-8 in human monocytes and aortic ECs and MCP-1 in human vascular smooth muscle cells. Homocysteine not only promotes the secretion of MCP-1 and interleukin-8 in human monocytes but also enhances the responsiveness of monocytes to MCP-1 in hyperhomocysteinemia patients. The present findings of a positive correlation of plasma homocysteine with SDF-1 levels and the elevation of SDF-1 in patient groups suggest that SDF-1 may play an important role in the pathogenesis of cardiovascular diseases. Furthermore, our results on human EC cultures showed that treatment with homocysteine concentrations characterizing mild to severe hyperhomocysteinemia (20 to 500 μmol/L) has stimulatory effects on SDF-1 expression. Therefore, hyperhomocysteinemia might provide an effective stimulus for SDF-1 accumulation in the arterial wall, thus promoting the recruitment of leukocytes and contributing to proinflammatory responses.

The endothelial lining of the vasculature plays an important role in sensing blood flow perturbations, leading to the modulation of gene expression in EC. At vessel bifurcations in an arterial tree, disturbed flow accompanied by LSS predisposes ECs to inflammation in which proinflammatory factors are involved; in contrast, laminar shear stress with a clear direction exerts atheroprotective effects. Our study showed that homocysteine-induced SDF-1 upregulation was inhibited in ECs subjected to HSS with a clear direction. Preexposure of ECs to a high level of shear stress inhibited homocysteine-induced signal transduction and SDF-1 expression. Previous studies demonstrated that shear stress also induced JNK activation in ECs and that this shear effect was dependent on the specific extracellular matrix. However, the shear-induced JNK activation seemed to be dependent on cell type and culture conditions. In our experiments, HUVECs cultured on fibronectin or collagen type I had similar inhibitory effect on homocysteine-induced SDF-1 expression. It is likely that shear stress modulates inflammatory responses by other mechanisms. Berk et al reported that shear stress-induced greater extent of ERK activation and that the ERK activation then repressed JNK activation in HUVECs, indicating that ERK pathway may mediate the shear stress suppression of JNK. Yoshizumi et al suggested that the possible mechanisms for this ERK-induced suppression include the JNK phosphatases and JNK interacting proteins. The tyrosine phosphatase activity of SHP-2 appears to be required for tumor necrotic factor α-induced signaling pathways. Inhibition of the phosphatase activity by shear stress represents a plausible mechanism by which shear stress modulates inflammatory factor–induced signal transduction. Therefore, elucidation of the mechanisms in homocysteine- and shear-mediated signal transduction requires further studies on ECs by manipulating activities of other upstream or downstream signaling proteins.

NO has been recognized to be an antiinflammatory molecule. It is produced by eNOS in response to physiological stimuli such as acetylcholine, thrombin, or shear stress. There is evidence in support of a role of NO in the regulating of gene expression in vascular cells. Endogenous NO production inhibited cytokine-induced expression of adhesion molecules as well as leukocyte adhesion. Exogenous addition of NO decreased MCP-1 expression in human ECs and also inhibited the expression of MCP-1 in smooth muscle cells. Previous observations showed that homocysteine decreases NO bioavailability in cultured ECs. In addition, homocysteine-induced EC apoptosis was inhibited by NO. In this study, administration of a SNAP significantly suppressed homocysteine-induced SDF-1 expression. Furthermore, NO produced from eNOS in response to shear stress has been shown to play a crucial role in shear-mediated antiatherogenic effects.
bioavailability of NO may contribute to thrombosis and atherosclerosis, because the prominent effects of EC-derived NO produced in response to shear stress include vessel relaxation and inhibition of cytokine-triggered platelet and monocyte adhesion.46 Our findings on the effects of L-NAME and eNOS siRNA on the shear-induced inhibition in SDF-1 expression in ECs are in concert with the hypothesis that NO may play a role in the shear-mediated suppression of proatherogenic factor–regulated genes. Preexposure of ECs to 4 hours of HSS inhibited homocysteine-induced SDF-1 expression, and this shear effect was blocked by treatment of L-NAME and transfection of eNOS siRNA. Thus, our findings also indicate that EC-derived NO may mediate homocysteine-induced SDF-1 expression in human ECs.

In summary, the present study demonstrates for the first time an increased expression of SDF-1 in ECs and in the circulating blood during hyperhomocysteinemia. This study has identified a unique molecular mechanism of homocysteine-induced JNK phosphorylation, Sp1 and AP-1 has identified a unique molecular mechanism of circulating blood during hyperhomocysteinemia. This study time an increased expression of SDF-1 in ECs and in the SDF-1 expression in human ECs.

References

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Subjects

We studied 32 patients, ages between 62 and 78, recruited from outpatients attending the Department of Cardiology of the St. Martin De Porres Hospital, Chiayi, Taiwan. Patients at high risks for cardiovascular events because of the presence of atherosclerotic disease or diabetes, or having at least two classical cardiovascular risk factors, including hypertension (blood pressure over 130/85 mm Hg), hypercholesterolemia (plasma cholesterol levels over 250 mg/dL), current smoker, body mass index (BMI) over 25 kg/m², waist over 90 cm in men or over 80 cm in women [1], were enrolled randomly from outpatient clinics.

In addition, we recruited 40 volunteers (ages between 57 and 86), who were admitted to the St. Martin De Porres Hospital for the purpose of routine physical examinations, as the normal subject group. None of the volunteers had ischemic heart disease, hypertension, stroke history, or peripheral vascular disease. Volunteers who smoked cigarettes, used alcohol or medications (hormonal replacement therapy, nonsteroidal anti-inflammatory drugs, corticosteroids, and anticoagulant drugs) were excluded from this normal subject group. The Ethics Committees of St. Martin De Porres Hospital and National Chiayi University approved the study protocol, and written informed consents were obtained from all patients before enrollment.

Materials

All culture materials were purchased from Gibco (Grand Island, NY, USA). Fibronectin (FN) and type I collagen (COLI) were purchased from BD Biosciences (San Diego, CA). PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) were purchased from Calbiochem (La Jolla, CA). Mithramycin A (an inhibitor of Sp1 binding) was purchased from Biomol Research
Laboratories (Plymouth Meeting, PA). S-nitroso-N-acetyl-penicillamine (SNAP) and \( N^\gamma \)-nitro-L-arginine methyl ester (L-NAME) were purchased from Alexis (San Diego, CA). The Sp1 and c-jun small interfering RNA (siRNA), mouse monoclonal antibodies (mAB) against extracellular signal-regulated kinase 2 (ERK2), JNK1, phospho-ERK, and phospho-JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against p38 and mouse monoclonal phospho-p38 antibody were purchased from Cell Signaling Technology (Beverly, MA). SDF-1 ELISA kit was obtained from R & D Systems (Minneapolis, MN). The ERK-, JNK-, p38-siRNA, endothelial nitric oxide synthase (eNOS)-siRNA, and control siRNA (scrambled negative control containing random DNA sequences) were purchased from Invitrogen (Carlsbad, CA). All other chemicals of reagent grade were obtained from Sigma (St Louis, MO).

**Measurement of plasma homocysteine**

Plasma homocysteine was measured on a morning blood specimen drawn after an overnight fast from an antecubital vein and collected in an EDTA tube, as previously described [2].

**SDF-1 enzyme-linked immunosorbent assay (ELISA)**

The levels of SDF-1 in the plasma and conditioned media were determined by using sandwich ELISA (sensitivity 18 pg/mL; R&D) according to manufacturer’s protocols, as previously described [3].

**Endothelial Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion. Human umbilical cords were rinsed and washed twice with PBS. The distal ends were clamped and the umbilical vein was filled with 0.1 % collagenase. After clamping the proximal end, the umbilical cord was incubated at 37°C for 10 min. The
vein was washed with PBS, followed by gentle massaging of the cords. The suspension of ECs collected was centrifuged for 10 min at 1200 \( \times \) g. The pellet was resuspended in M199 supplemented with penicillin/streptomycin and 20% FBS, after which the cells were plated onto cell culture dishes. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO\(_2\). Non-adherent cells and red blood cells were removed after 1 or 2 days by changing the medium, and adherent cells were kept static with the culture medium changed every 3 days [3]. HUVECs of passage 2 were used for experiments.

Human aortic endothelial cells (HAECs) were purchased from Cambrex Bio Science (Walkersville, MD) and were used before the passage 4. Cells were cultured in M199 containing 15% FBS at 37°C and 5% CO\(_2\).

**Shear Stress Experiment**

The glass slides were pre-coated with FN or COLI at 37°C for 1 h, and ECs were then seeded on the slides for 24 h. The glass slide with cultured ECs was mounted in a parallel-plate flow chamber characterized and described in detail [4]. The chamber was connected to a perfusion loop system and kept at 37°C in a temperature-controlled enclosure. The perfusate was maintained at pH 7.4 by continuous gassing with a humidified mixture of 5% CO\(_2\) in air. Two levels of fluid shear stress (\(\tau\)) were generated on the cells by flow: a low shear stress (LSS) at 0.5 dyn/cm\(^2\) and a high shear stress (HSS) at 20 dyn/cm\(^2\), unless otherwise noted, using the formula \(\tau = 6\mu Q/wh^2\), where \(\mu\) is the dynamic viscosity of the perfusate, \(Q\) is the flow rate, and \(h\) and \(w\) are the channel height and width, respectively.

**Real-time quantitative PCR.**

Total RNA preparation and the RT reaction were carried out as described previously [5]. PCRs were performed using an ABI Prism 7900HT according to the
manufacturer's instructions. Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems). The designed primers in this study were: SDF-1 forward primer, 5'-GCC GCT CCC AAC TTA CAG AA-3'; SDF-1 reverse primer, 5'- CCC ATC AAC GGT CTG GAA CT-3'; 18S rRNA forward primer, 5'-CGG CGA CGA CCC ATT CGA AC-3', 18S rRNA reverse primer, 5'-GAA TCG AAC CCT GAT TCC CCG TC-3'. RNA samples were normalized to the level of 18S rRNA. SDF-1 primer pairs had at least one primer crossing an exon-exon boundary. The real-time PCR was performed in triplicate in a total reaction volume of 25 µL containing 12.5 µL of SYBR Green PCR Master Mix, 300 nM forward and reverse primers, 11 µL of distilled H₂O, and 1 µL of cDNA from each sample. Samples were heated for 10 min at 95°C and amplified for 40 cycles of 15 sec at 95°C and of 60 sec at 60°C. Quantification was performed using the 2^{−ΔΔCt} method [5], where Ct value was defined as threshold cycle of PCR at which amplified product was detected. The ΔCt was obtained by subtracting the housekeeping gene (18S rRNA) Ct value from the Ct value of the gene of interest (SDF-1). The present study used ΔCt of control subjects as the calibrator. The fold change was calculated according to the formula $2^{−ΔΔCt}$, where ΔΔCt was the difference between ΔCt and the ΔCt calibrator value (which was assigned a value of 1 arbitrary unit).

**Western Blot Analysis**

ECs were lysed with a buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). The total cell lysate (50 µg of protein) was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (12% running, 4% stacking) and analyzed by using the designated antibodies and the Western-Light chemiluminescent detection system (Bio-Rad, Hercules, CA), as previously described [6].
**Reporter gene construct, siRNA, transfection, and luciferase assays**

SDF-1 promoter constructs contain -1100/+122, -630/+122, -430/+122, -395/+122, -214/+122, -121/+122, and -20/+122 of SDF-1 5'-flanking DNA linked to the firefly luciferase reporter gene of plasmid pGL4 (Promega, Madison, WI). DNA plasmids at a concentration of 1 mg/ml were transfected into HUVECs by using Lipofectamine (Gibco). The pSV-β-galactosidase plasmid was cotransfected to normalize the transfection efficiency. The cells were kept as static controls or subjected to shear stress experiments 48 h after transfection. For siRNA transfection, HUVECs were transfected with the designated siRNA by using a RNAiMAX transfection kit (Invitrogen).

**Adenoviral Infection.**

Dominant-negative mutants of ERK (DN-ERK), JNK (DN-JNK), and p38 (DN-p38) were produced as previous described [7]. Adenoviruses bearing cDNAs for DN-ERK, DN-JNK, and DN-p38 were prepared using the ViraPower Adenovirus Expression System (Invitrogen, Carlsbad, CA) according to the manufacture’s instruction. Briefly, the cDNA was subcloned into the pDONR (Invitrogen) vector by using BP Clonase (Invitrogen) and then shuttled into pAd/CMV/V5-DEST vector (Invitrogen) using LR Clonase (Invitrogen). The resultant plasmid was purified, sequenced, and transfected into the 293A producer cell line (ViraPower System, Invitrogen) that supplies the E1 proteins necessary to generate adenovirus. Control adenoviruses bearing green fluorescent protein (Ad-GFP) were prepared as previous described [6]. For adenoviral infection, HUVECs were infected with the indicated adenovirus (MOI = 50) for 12 h, and then further incubated for 48 h with fresh medium before treatment with the reagents.

**Sp1 and AP-1 transcription factor assays (TF ELISA assays).**
Nuclear extracts of cells were prepared as previously described [5]. Equal amounts of nuclear extracts were used for quantitative measurements of Sp1 and AP-1 activation using commercially available ELISA kits (Panomics, Redwood City, CA) that measure Sp1- and AP-1-DNA binding activities.

**Chromatin immunoprecipitation (ChIP).**

After cross-linking with 1% formaldehyde, the cells were centrifuged and then resuspended in a lysis buffer for three times of sonication of 15 sec each. Supernatants were recovered by centrifugation. Aliquots of the precleared sheared chromatin were then immunoprecipitated using 2 μg antibodies against IgG, Sp1, or c-Jun. The resulting DNA was used for PCR analysis, and the amplified DNA fragments were visualized on an agarose gel [5]. PCR was performed with the following primers that amplify the part of the human SDF-1 promoters that contain the Sp-1 binding sites: 5'-CAC AGA GGG AGC GGA GGA GG-3' and 5'-CGG GCG CTT TAG AGG GGA GA-3', and AP-1 binding sites: 5'-CAC GCA CAG AAA GC A GGA CC-3' and 5'-TGG CGG GAA CTG AAT GAG AA-3'.

**Statistical Analysis**

The results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was determined by using an independent Student t-test for two groups of data and analysis of variance (ANOVA) followed by Scheffe’s test for multiple comparisons. P values less than 0.05 were considered significant.

**References**


D. Rapid measurement of total plasma homocysteine by HPLC. *Clin Chim Acta.* 2003; 331:19-23.


Figure S1. Relationships between plasma levels of homocysteine and SDF-1 in all subjects (n = 72). Black circles: patients recruited from outpatients at high risk for cardiovascular events. White circles: normal volunteers from routine physical examinations.
Figure S2. Stimulation with homocysteine induces HUVECs to increase their phosphorylation of ERK (A), JNK (B), and p38 (C). HUVECs were kept as controls (CL) or stimulated with 100 μM homocysteine for the times indicated, and the phosphorylations of ERK, JNK, and p38 were determined by using Western blot analysis. Phosphorylated ERK, JNK, and p38 levels are presented as band densities (normalized to total protein levels) relative to CL. The results are mean ± SEM from 3 independent experiments. *P < 0.05 versus control EC (CL).
Figure S3. HUVECs were kept as controls (CL) or presheared at HSS (20 dyn/cm²) for 1 ~ 4 h before homocysteine stimulation. Static ECs were stimulated with homocysteine without preshearing (static). Data are presented folds of control ECs (CL), mean ± SEM. "P < 0.05 versus static ECs (static) with homocysteine stimulation.
Figure S4. HUVECs cultured on FN- or COLI-coated glass were presheared at HSS (20 dyn/cm²) for 4 h (HSS/FN or HSS/COLI) before homocysteine stimulation. Static ECs were ECs cultured on FN- or COLI-coated glass and stimulated with homocysteine without preshearing (static). Data are presented folds of control ECs (CL), mean ± SEM. *P < 0.05 versus CL. #P < 0.05 versus static ECs (static/FN or static/COLI) with homocysteine stimulation.
Figure S5. HUVECs were kept as controls (CL) or presheared at HSS (20 dyn/cm²) or LSS (0.5 dyn/cm²) for 4 h before homocysteine stimulation. Static ECs were stimulated with homocysteine without preshearing (static). The SDF-1 p1010-Luc activity after 4 h homocysteine stimulation was determined by luciferase assay normalized to β-galactosidase. Data are presented folds of control ECs (CL), mean ± SEM. *P < 0.05 versus CL. †P < 0.05 versus static ECs (static) with homocysteine stimulation.
**Figure S6.** HUVECs were kept as controls (CL) or presheared at HSS (20 dyn/cm²) or LSS (0.5 dyn/cm²) for 4 h before homocysteine stimulation. Static ECs were stimulated with homocysteine without preshearing (static). Sp1 (A) and AP-1 (B) activation after 2 h homocysteine stimulation in HUVECs was performed by TF ELISA assay. Data are presented folds of control ECs (CL), mean ± SEM. *P < 0.05 versus CL. #P < 0.05 versus static ECs (static) with homocysteine stimulation.
Figure S7. Schematic representation of the inhibitory effects of shear stress and the signaling pathway regulating the expressions of SDF-1 in ECs in response to homocysteine stimulation.