Homocysteine Inhibits Arterial Endothelial Cell Growth Through Transcriptional Downregulation of Fibroblast Growth Factor-2 Involving G Protein and DNA Methylation


Abstract—Homocysteine (Hcy) contributes to atherogenesis and angiostasis by altering the phenotype of arterial endothelial cells (ECs). The present study was aimed at elucidating potential mechanisms by which Hcy can slow EC proliferation and induce EC apoptosis, thereby disrupting endothelial integrity. Given the strong mitogenic and antiapoptotic properties of fibroblast growth factor (FGF)2, we examined whether Hcy can modulate its expression. In cultured human coronary and bovine aortic ECs, Hcy exerted time- and concentration-dependent (0 to 500 μmol/L) reduction of the mRNA and protein levels of FGF2, whereas vascular endothelial growth factor expression was not affected until Hcy reached a proapoptotic 500 μmol/L. By testing a panel of signal transduction inhibitors, we found that the Hcy-induced downregulation of FGF2 was specifically attenuated by pertussis toxin, an inhibitor of Gi protein signaling. Hcy induced cell cycle arrest at the G1/S transition and increased TUNEL-positive apoptotic cells in a graded manner. These effects were effectively counteracted by exogenous FGF2. Reporter gene assays showed that Hcy downregulated FGF2 by transcriptional repression of the gene promoter encompassed in a CpG dinucleotide-rich island. This region was heavily methylated at the cytosine residues by Hcy despite decreased methylation potential (S-adenosylmethionine to S-adenosylhomocysteine ratio). Normal levels of FGF2 transcription were restored to ECs simultaneously exposed to Hcy and 5-aza-deoxycytidine. We conclude that homocysteine disrupts the growth and survival of ECs through a G protein–mediated pathway associated with altered promoter DNA methylation and the transcriptional repression of FGF2. (Circ Res. 2008;102:933-941.)

Key Words: homocysteine ■ endothelial cells ■ growth factors ■ transcriptional regulation ■ DNA methylation

O cculsive vascular diseases profoundly contribute to morbidity and mortality in industrialized populations, prompting exhaustive approaches to identifying and characterizing potential etiologic factors. One potential factor, an elevated plasma homocysteine (Hcy) concentration, arises from the abnormal metabolism of methionine from dietary protein. Nearly 40 years ago, a series of case studies led to the proposal that despite different metabolic deficiencies causing homocystinuria, the common excess metabolite homocysteine induced premature atherosclerosis and fatal thrombosis.1,2 Controversy remains over whether hyperhomocysteinemia (HHcy) is an independent or a conditional risk factor or simply a marker of cardiovascular disease.3–6 Mild to moderate HHcy has been detected in 12% to 47% of patients exhibiting coronary, cerebral, and peripheral arterial disease.7 Abundant clinical and epidemiological studies have associated HHcy with heightened risks of arterial diseases, yet not all prospective studies have reached this conclusion. Recent metaanalyses have indicated that increases in plasma Hcy concentration of merely 3 to 5 μmol/L can raise the risk for myocardial infarction, stroke, or venous thromboembolism by 10% to 27%.7–9 Likewise, some, yet not all, clinical trials testing Hcy-lowering strategies have found a diminished recurrence of cardiovascular events such as restenosis, stroke, or venous thromboembolism.10–12 Atherosclerosis/angiostasis partially stems from the injury or phenotypic alteration of endothelial cells (ECs), the cells in the frontline against vascular disturbance. The histological examination of hyperhomocysteinemic (HHcyc) rats revealed an increased recruitment of monocytes to aortic endothelium.
accompanied by elevated immunostaining for monocyte chemotactic protein-1, vascular cell adhesion molecule-1, and E-selectin. Studies in humans and several transgenic murine models of HHcy have uncovered widespread arterial endothelial dysfunction in cerebral, mesenteric, and cremasteric arterioles. Endothelial dysfunction in HHcy mice has been associated with a decreased bioavailability of endothelial NO through diminished eNOS activity; however, the induction of oxidative stress does not consistently represent a Hcy-specific mechanism of endothelial damage. Recently, more and more data have accumulated that inhibition of angiogenesis in HHcy subjects can be attributed to EC apoptosis and impaired angiogenic response involving EC proliferation, migration, and tube formation. Although the angiostatic effect of Hcy has been suggested to arise from the decreased expression of angiogenic growth factors, the underlying mechanism has not been thoroughly characterized.

The specific conversion of Hcy to S-adenosyl Hcy (SAH) represents another mechanism capable of perturbing EC phenotype. Altered methylation patterns associated with increased levels of SAH have been reported to modulate protein function and the transcriptional control of GC-rich promoters. In addition, Hcy conversion to SAH was associated with decreases in p21ras carboxymethylation, extracellular signal-regulated kinase \( \frac{1}{2} \)-mediated signaling and cyclin A expression. Altogether, ECs treated with clinically relevant concentrations of Hcy exhibited decreased DNA synthesis and delayed progression through the cell cycle at the G1/S transition.

We previously demonstrated that copper-oxidized LDL (oxLDL) and L5, an electronreceptive LDL isolated from hypercholesterolemic or type 2 diabetic human plasma, can inhibit EC proliferation and angiogenesis. These angiostatic effects of oxLDL and L5 were accompanied by the downregulation of fibroblast growth factor (FGF2) and inhibited by FGF2 supplementation. In contrast, vascular endothelial growth factor (VEGF) failed to attenuate oxLDL-induced angiostasis. We also found that a pertussis toxin-sensitive G protein pathway was involved in the oxLDL-mediated downregulation of FGF2. The ability of Hcy to inhibit EC proliferation and angiogenesis resembled that of our modified LDLs. On the basis of this finding, we confirmed the effects of clinically significant Hcy concentrations on EC growth and survival before mechanistic studies investigating whether Hcy can alter the expression of FGF2 or VEGF through specific regulatory pathways.

Materials and Methods

The chemicals and reagents used in this study and all experimental techniques, including cell cultures and treatment protocols, inhibitors of signal transduction pathways, DNA synthesis, FGF2 and VEGF ELISA, cell viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, fluorescence-activated cell-sorting analysis, TUNEL assays, real-time PCR, cell transfection and reporter gene assays, liquid chromatography–tandem mass spectrometry (MS/MS) measurement for S-adenosylmethionine (SAM) and SAH and bisulfite genomic DNA sequencing were described in the online data supplement, available at http://circres.ahajournals.org.

Figure 1. Effects of Hcy on DNA synthesis, cell viability, and cell death in cultured BAECs. A, Dose-dependent effects. Cells were treated with increasing concentrations of Hcy for 24 hours, and DNA synthesis (\( \text{ng} / \text{ml} \)), cell viability (\( \text{mg} / \text{ml} \)), and cell death (\( \% \)) were assessed. Results were means\( \pm \)SEM (n=3). *P<0.05 vs PBS control. B, Time-dependent effects. BAECs \((10^6)\) were incubated with PBS (\( \bullet \)), or 50 \( \mu \text{mol}/\text{L} \) Hcy (\( \square \)) or 100 \( \mu \text{mol}/\text{L} \) Hcy (\( \bigtriangledown \)), or 100 \( \mu \text{mol}/\text{L} \) cysteine (\( \bigcirc \)) for 0 to 96 hours, followed by cell viability assay. Values are means\( \pm \)SEM (n=3). **P<0.01, ***P<0.001 vs corresponding untreated controls. B, Time-dependent effects. BAECs \((5 \times 10^6)\) were incubated with PBS (\( \bullet \)), or 50 \( \mu \text{mol}/\text{L} \) Hcy (\( \square \)) or 100 \( \mu \text{mol}/\text{L} \) Hcy (\( \bigtriangledown \)), or 100 \( \mu \text{mol}/\text{L} \) cysteine (\( \bigcirc \)) for 0 to 96 hours, followed by cell viability assay. Values are means\( \pm \)SEM (n=3). **P<0.01 vs PBS control.

Statistical Analysis

The significance of the differences between group means was assessed by a modified t test (Bonferroni test) for multiple comparisons. ANOVA followed by Scheffé’s test for significance were used to compare concentration- and time-dependent responses. A probability value of <0.05 was considered significant. Results were expressed as means\( \pm \)SEM values.

Results

Hcy Decreased DNA Synthesis and Cell Viability

The exposure of bovine aortic ECs (BAECs) to Hcy for 24 hours decreased DNA synthesis in a concentration-dependent manner (Figure 1A). Compared with PBS control, 50 and 100 \( \mu \text{mol}/\text{L} \) Hcy reduced \( \text{H} \)-thymidine incorporation by 20% and 30%, respectively, without increasing the number of dead cells. Similarly, MTT assay revealed that treatment with 100 \( \mu \text{mol}/\text{L} \) Hcy decreased cell viability by 40% (Figure 1A). At concentrations >100 \( \mu \text{mol}/\text{L} \), Hcy not only decreased DNA synthesis by 70% but also greatly reduced cell viability and markedly increased dead cell population (Figure 1A). Consistent with the inhibitory effect on \( \text{H} \)-thymidine incorporation, Hcy markedly decreased cell viability in a time- and concentration-dependent manner, whereas cysteine had no
effect (Figure 1B). When Hcy was removed from the medium at 24 hours, cell viability was restored gradually through 96 hours (Figure I in the online data supplement).

**Hcy Reduced FGF2 Expression in BAECs and Human Coronary Arterial ECs**

Because FGF2 is a potent mitogenic and antiapoptotic protein, we examined whether Hcy may affect its endogenous expression. As assayed by ELISA, Hcy decreased intracellular FGF2 in a concentration-dependent (25 to 500 μmol/L at 24 hours) and time-dependent (24 to 96 hours at 100 μmol/L) manner in BAECs (Figure 2A, top graphs). These concentration- and time-dependent effects of Hcy on FGF2 reduction were species independent because human coronary arterial ECs (HCAECs) had similar response to Hcy (Figure 2A, bottom graphs). FGF2 mRNA levels were determined by real-time PCR (Figure 2B). In both BAECs and HCAECs treated with 100 μmol/L Hcy for 24 hours, FGF2, but not FGFR1, mRNA was decreased by 40% relative to PBS control, compatible with the extent of FGF2 protein reduction assayed by ELISA. Cysteine and methionine, 2 important sulfur-containing amino acids derived from Hcy metabolism, had no effect.

In comparison with FGF2, the intracellular VEGF protein levels were not changed until Hcy reached a proapoptotic concentration of 500 μmol/L at 24 hours in BAECs (supplemental Figure IIA) and HCAECs (supplemental Figure IIB). There was no reduction in intracellular VEGF through 96 hours with 100 μmol/L Hcy. In contrast to FGF2 mRNA, which was reduced by 100 μmol/L Hcy, VEGF mRNA levels were not affected at this physiological concentration. When Hcy was raised to 500 μmol/L, both FGF2 and VEGF mRNA were remarkably reduced as a result of cell death (supplemental Figure IIC).

**Involvement of G Protein Signaling in Hcy-Induced FGF2 Downregulation**

The signal transduction pathways of Hcy-mediated FGF2 downregulation were investigated using pharmacological inhibitors (Figure 2C). At 24 hours Hcy (100 μmol/L) decreased intracellular FGF2 protein by ~40% compared with PBS. This inhibitory effect was remarkably blocked in cells incubated with the G protein inhibitor pertussis toxin (100 ng/mL) for 18 hours before Hcy exposure. In contrast, pretreating the cells with the MEK/MAPK inhibitor PD-98059 (20 μmol/L) for 18 hours or the protein kinase C inhibitor Ro-31 to 8220 (3.5 μmol/L) for 1 hour failed to counteract the inhibitory effects of Hcy on FGF2 protein levels. Blocking the cAMP-dependent protein kinase with HA-1004 (10 μmol/L) for 18 hours or chelating the intracellular Ca2+ with BAPTA-AM (16 μmol/L) for 1 hour also failed to attenuate the effect of Hcy.

**Effects of FGF2 on Hcy-Induced Cell Cycle Arrest and Apoptosis**

Given that Hcy can inhibit the expression of FGF2 in ECs, we examined whether providing these growth factors could counteract the effects of Hcy on DNA synthesis and cell cycle progression. In the absence of Hcy, both FGF2 and VEGF-165 (50 ng/mL each) significantly increased DNA replication. With 100 μmol/L Hcy, however, only FGF2 completely restored normal levels of DNA synthesis (Figure 3A). Thus, Hcy did not impair the ability of exogenous FGF2 to promote DNA replication. Conversely, in the presence of Hcy, VEGF-165 did not significantly increase DNA replication. The exposure of BAECs to 100 μmol/L Hcy significantly increased the percentage of cells retained in the G1 phase of the cell cycle (from 60% to 76%; P<0.05; n=3) (Figure 3B and supplemental Table II), with a concomitant decrease in cells entering the S phase and no effect on the G2/M transition. As anticipated, the simultaneous treatment of BAECs with FGF2 prevented Hcy-induced G1 arrest, whereas VEGF-165 was ineffective. This could reflect the noninvolvement of VEGF in this effect of Hcy (supplemental Figure II).

The effects of FGF2 and VEGF on Hcy-induced EC apoptosis were evaluated using TUNEL assay (Figure 4). BAECs treated with graded concentrations of Hcy for 24 hours revealed a dose-dependent increase in the percentage of TUNEL-positive cells. At 100 μmol/L, Hcy had no significant effect on EC apoptosis compared with PBS control (7.2±5.3% vs 2.0±1.1%; n=3). At 200 and 500 μmol/L, the TUNEL-positive cells represented 38.2±11.4% (P<0.05; n=3) and 78.2±13.1% (P<0.01; n=3), respectively, compared with PBS control. When FGF2 was added to BAECs cultured in 500 μmol/L Hcy, the TUNEL-positive cells decreased markedly (24.7±12.8% vs 78.2±13.1%; P<0.01; n=3). In comparison, VEGF moderately attenuated the apoptosis-inducing activity of high-dose Hcy (45.6±9.3% vs 78.2±13.1%; P<0.05; n=3).

**Transcriptional Regulation of FGF2**

Human FGF2 genomic DNA fragments were generated by PCR and inserted into the firefly luciferase vector pGL3. The promoter activities of these constructs were evaluated in HCAECs in the presence or absence of 100 μmol/L Hcy (Figure 5). Compared with pGL3-basic, the −968/+43 construct increased luciferase activity by 40-fold. Deletion from −968 to −127 resulted in an additional 3-fold increase, suggesting the removal of negative regulatory elements in this region. Further deletion from −127 to −101 modestly reduced promoter activity. However, further deletion to −34 resulted in a >80% decrease in FGF2 promoter activity, indicating that a basal promoter was located between −100 and −35. The addition of 100 μmol/L Hcy, but not cysteine or methionine, reduced reporter activities by ~30% to 40% from constructs spanning −968/+43, −126/+43, and −100/+43 but not in −34/+43 or +24/+179 lacking crucial promoter sequences. These results indicate a basal promoter driving FGF2 transcription that can be repressed by the treatment of HCAECs with Hcy.

**Methylation Status of FGF2 Gene**

An analysis of the human FGF2 gene identified an 1877-bp CpG island spanning from −532 in the 5′-flanking region and extending through exon 1 into the first intron. This portion of the human FGF2 gene encompasses the Hcy-responsive basal promoter, as well as clusters of CpG dinucleotides predicted to be methylation sensitive (supplemental Figure IIIA). To investigate whether methylation...
participates in Hcy-induced FGF2 downregulation, the methylation status of CpG dinucleotides in the FGF2 promoter was characterized by bisulfite genomic DNA sequencing in Hcy-treated HCAECs (supplemental Figure IIIB). Thirty-one CpG dinucleotides, numbers 1 to 31, in the FGF2 5′-flanking region were analyzed using 2 pairs of primers (CpG primer 1S/1AS; CpG primer 2S/2AS) designed to amplify the FGF2 promoter region. None of the 31 cytosine residues was methylated in the PBS control cells. In contrast, cytosine residue numbers 9 to 28 were methylated in the presence of Hcy.

Figure 2. Effects of Hcy on FGF2 expression. A, Dose-dependent and time-dependent effects on FGF2 protein levels in BAECs (top graphs) or HCAECs (bottom graphs). Intracellular FGF2 protein levels were assayed by ELISA. Cells treated with PBS for 96 hours were used as control. Values are means ± SEM (n=3). *P<0.05, **P<0.01 vs PBS control. B, Measurement of mRNA by real-time PCR. Cells were incubated with PBS or 100 μmol/L each of Hcy, cysteine, or methionine (Met) for 24 hours, and total RNA was subjected to real-time PCR analysis with specific primers for FGF2 and FGFR1. Values are means ± SEM (relative to PBS-treated samples) after normalization to β-actin (n=4). *P<0.05 vs PBS control. C, Effects of signal transduction inhibitors on Hcy-induced reduction in FGF2 assessed by ELISA in cultured BAECs. Cells were incubated with 100 μmol/L Hcy for 24 hours alone or with preceding exposure to signaling pathway modifiers as indicated, according to the protocols described in the text. PBS was used as a control. Values are means ± SEM (n=3). *P<0.05 vs PBS.
100 μmol/L Hcy, and increasing Hcy to 500 μmol/L resulted in methylation of all 31 cytosine residues (supplemental Figure IIIB). These data indicate that Hcy promotes methylation of the CpG dinucleotides in the FGF2 promoter and thus represses FGF2 transcriptional activity in ECs.

Reporter gene assay was performed to compare transcriptional activities of FGF2 in HCAECs cultured in the presence of Hcy with or without the methylation inhibitor 5-aza-deoxycytidine (5-aza-dC). As shown in Figure 6A, the promoter activities of −969/+43 and −126/+43 constructs

Figure 3. Opposing effects of Hcy and FGF2 on DNA synthesis and cell cycle transitions. A, BAECs (1×10⁶) were treated with 100 μmol/L Hcy, 50 ng/mL FGF2, and 50 ng/mL VEGF-165, alone or in combination. DNA synthesis was assessed by [³H]-thymidine incorporation in cells after 24 hours of treatment. Values are means ± SEM (n=3). *P<0.05, **P<0.01 vs PBS control. B, BAECs were incubated with 100 μmol/L Hcy in the presence or absence of 50 ng/mL FGF2 or 50 ng/mL VEGF for 24 hours before fluorescence-activated cell sorting analysis. The figure is representative of 3 separate experiments with similar results. Distributions of the cells in the G₁, S, and G₂/M phases are shown. Values are tabulated in supplemental Table II.

Figure 4. Apoptotic effects of Hcy. A, BAECs were incubated with 100, 200, or 500 μmol/L Hcy for 24 hours in the presence or absence of 50 ng/mL FGF2 or 50 ng/mL VEGF, and apoptosis was assessed by TUNEL assay. Control cells were treated with PBS. B, The percentages of TUNEL-positive cells are expressed as means ± SEM (n=3). *P<0.05, **P<0.01 vs PBS control; †P<0.05, ††P<0.01 vs the Hcy-treated (500 μmol/L) sample.
containing an Hcy-response element were reduced by Hcy; addition of 5-aza-dC significantly attenuated the Hcy effect. Cellular FGF2 mRNA levels in the presence of Hcy with or without 5-aza-dC were compared across HCAECs by real-time PCR (Figure 6B). Consistent with reporter gene assays, Hcy alone resulted in a ~40% reduction in FGF2 mRNA. The addition of 5-aza-dC to Hcy-treated HCAECs increased cellular FGF2 mRNA to normal levels. These results indicate that DNA methylation is an important mechanism mediating FGF2 transcription and cell proliferation in HCAECs.

**Discussion**

Our data showing that Hcy can specifically downregulate FGF2 expression in arterial ECs reveal a novel mechanism whereby HHcy can be angiostatic. The schematic in Figure 7 outlines a signaling pathway through which Hcy can impair endothelial proliferation/survival by FGF2 suppression. As an important risk factor for atherosclerosis, Hcy can therefore exhibit synergistic EC toxicity with oxLDL or L5 by a shared pathway related to FGF2. Both FGF2 and VEGF contribute to angiogenesis by promoting EC proliferation, migration, and tube formation. These processes were inhibited in BAECs exposed to Hcy at a supraphysiological concentration (5 mmol/L). This observation highlighted the need to confirm the effects of Hcy on EC proliferation and viability within a clinically significant Hcy concentration range before mechanistic investigation. HHcy has been defined as a plasma concentration exceeding 14 μmol/L and is considered severe at levels beyond 100 μmol/L, arising from rare metabolic defects of thiol amino acid metabolism. Notwithstanding, mild HHcy (15 to 30 μmol/L) has been associated with increased risks for a variety of occlusive vascular diseases. Thus, our experiments were conducted by incubating BAECs or HCAECs with Hcy ranging from 25 to 500 μmol/L. We have shown here that the treatment of ECs with Hcy concentrations characterizing mild to moderate HHcy (25–100 μmol/L) decreased DNA replication without increasing apoptosis. A structural analog of Hcy, cysteine, did not mimic the effects of Hcy on EC viability or FGF2 expression, indicating that receptor-1 in endothelial and mononuclear cells and increase oxLDL toxicities. Collectively, our results support cross-talk between Hcy and oxLDL and signify the need to upregulate or introduce FGF2 to counteract their pathological effects on ECs.

![Figure 5. Effects of Hcy on FGF2 transcription in cultured HCAECs. FGF2 reporter gene constructs and reporter gene assay are shown. Different lengths of human FGF2 5' flanking sequences were fused to luciferase gene in pGL3-basic vector. The transcription start site is indicated as +1. All constructs were cotransfected with phRL-TK (internal control) into HCAECs and incubated in the presence or absence of 100 μmol/L each of Hcy, cysteine, or methionine (Met) for 24 hours. Luciferase activities were expressed as fold increases over pGL3-basic. Values are means±SEM (n=3 to 5). *P<0.05 vs PBS control.](http://circres.ahajournals.org/)

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the effects of Hcy occurred independently of thiol reactivity. Hcy-induced changes in EC phenotype were accompanied by decreased levels of intracellular FGF2 but not VEGF at more physiological, nonapoptotic concentrations (≤ 100 μmol/L). On the contrary, high-dose Hcy at proapoptotic levels (500 μmol/L) decreased intracellular FGF2 as well as VEGF, consistent with the activation of degradative pathways and cell death (Figures 1 and 7). These observations offer a consistent with the activation of degradative pathways and death (Figures 1 and 7). These observations offer a potential explanation for the severe damage or denudation of the arterial endothelium in homozygous homocystinurics or experimental primates infused with large amounts of Hcy.2,35

Several other derivatives of Hcy, such as Hcy thiolactone, S-nitroso Hcy, or SAH, affect vascular homeostasis. In BAECs and HCAECs treated with 100 μmol/L Hcy, we found reduced levels of FGF2 protein and mRNA. Thus, our study focused on SAH because this metabolite has been shown to affect gene expression at the epigenetic level by shifting the cellular methylation potential.41 Our discovery that Hcy-induced FGF2 downregulation in arterial ECs. The transcriptional regulation of FGF2 mRNA by oxLDL is also shown for comparison.36,37,38 Transcription start site of FGF2 gene is indicated as +1. Methylation of the cytosine residues of CpG dinucleotides is indicated by CH3.

We have shown here that Hcy downregulated FGF2 through the transcriptional repression of its promoter. Hcy reportedly modulates the expression of multiple genes in different cell types through changes in transcription factor activity47 or DNA methylation.25 This prompted our analysis of the Hcy-responsive basal promoter identified here from −100 to −34 using the TRANSFAC database. Although searches failed to reveal NF-κB sites, we identified GC boxes with CpG islands that can be regulated by methylation.38 In cultured cells, Hcy has been shown to induce gene-specific DNA hypomethylation regulating the expression of Hcy-induced endoplasmic reticulum protein (HERP) and an imprinted gene H19.39,40 However, some reports indicate that hypomethylation can be followed by the global hypermethylation of DNA through mechanisms not yet understood.39 In fact, leukocytic DNA from an SAH hydrolase-deficient individual paradoxically has been found to be hypermethylated.41 Our discovery that Hcy-induced FGF2 downregulation could be attenuated by 5-aza-dC, a well-known demethylating reagent,42 also supported the paradoxical hypermethylation of the FGF2 promoter as illustrated in Figure 7. Among the genes previously shown to be upregulated in Hcy-treated human umbilical vein endothelial cells, the bifunctional enzyme NAD-dependent methyltetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase (NMDMC) has been identified with the ability to increase the production of 5-methyltetrahydrofolate required for Hcy remethylation.43 Thus, ECs may be able to adjust to mediated cytotoxities (Table). Indeed, increasing Hcy concentrations were associated with a dose-dependent increase in intracellular SAH but a concomitant decrease in methylation potential (Table), which is correlated with the observed Hcy effects (Figure 1). These culture conditions, with EHNA and adenosine in the medium, were required to observe the adverse effects of 25 to 500 μmol/L Hcy on EC growth and survival and the downregulation of FGF2. Hcy alone (up to 500 μmol/L) did not induce these EC toxicities (data not shown).

Figure 6. FGF2 promoter analysis. A. Reporter gene assay. The reporter constructs were cotransfected with pGL3-TK (internal control) into HCAECs, followed by incubation with Hcy 100 μmol/L in the presence or absence of 5-aza-dC or pertussis toxin (PTX). Luciferase activities were expressed as fold increases over pGL3-basic. Values are means ± SEM (n=3–5). *P<0.05 vs PBS control. B. Demethylation assay. HCAECs were incubated with PBS or Hcy 100 μmol/L in the presence or absence of 5-aza-dC (0.4 μg/mL) or pertussis toxin. Total RNA was subjected to real-time PCR analysis with specific primers for FGF2. Values are means ± SEM (n=3) relative to PBS control. *P<0.05 vs PBS sample.

Figure 7. Schematic illustration of regulatory pathways of Hcy-induced FGF2 downregulation in arterial ECs. The transcriptional regulation of FGF2 mRNA by oxLDL is also shown for comparison.36,37,38 Transcription start site of FGF2 gene is indicated as +1. Methylation of the cytosine residues of CpG dinucleotides is indicated by CH3.
elevated levels of SAH by upregulating enzymes capable of compensatory remethylation reactions promoting DNA hypermethylation.

Our findings present important clinical implications because angiogenesis plays a crucial role in different physiological and pathological processes such as embryonic development, wound repair, tumor growth, and atherosclerosis. FGFR2 and VEGF are not necessarily functionally redundant during angiogenesis accompanying atherosclerosis, such as plaque neovascularization and collateral arteriogenesis. In the present study, it is obvious that FGFR2 is more susceptible to Hcy than VEGF in terms of gene expression: FGFR2 was suppressed at as low as 100 μmol/L, but VEGF reduction did not occur until Hcy reached a proapoptotic concentration of 500 μmol/L. This explains why VEGF failed to attenuate cell cycle arrest but could ameliorate apoptosis when Hcy concentrations were raised from antiproliferative to antisurvival levels (Figure 7).

Moreover, our findings that Hcy-mediated programmed cell death could be effectively ameliorated by either FGFR2 or VEGF also support the involvement of two distinct apoptotic pathways in ECs as previously reported. Consistent with these results, it is well known that Hcy activates the unfolded protein response and induces apoptosis through a Fas-mediated extrinsic pathway, which could presumably be prevented by VEGF. In contrast, FGFR2 protects against EC apoptosis through an intrinsic pathway independent of VEGF signaling. Altogether, these reports suggest a complex interplay between FGFR2 and VEGF signaling in Hcy metabolism that can incur endothelial phenotypic changes.

In summary, compensatory arteriogenesis has been shown to be orchestrated by either FGFR2 or VEGF. oxLDL, L5, or Hcy may inhibit this process by repressing FGFR2 expression in ECs through a species-independent manner. Understanding the mechanisms by which Hcy and modified LDL, including oxLDL and L5, can inhibit angiogenesis may suggest novel therapeutic strategies to upregulate angiogenesis, thereby promoting the perfusion of ischemic tissues, such as for collateral arteriogenesis or optimal embryonic development, or to downregulate angiogenesis and attenuate cancer.

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### Disclosures

None.

### References


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Online Materials and Methods

Materials
The following materials were purchased: recombinant human FGF2 and VEGF-165 and their monoclonal antibodies (Upstate Biotechnology); restriction endonucleases (New England Biolabs); $^3$H-thymidine (Moravek Biomedicals); radionucleotides (DuPont NEN); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *erythro*-9(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA), *D,L*-homocysteine, cysteine and 5-aza-deoxycytidine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), $[^3]$H$_3$]-SAM, ammonium acetate, formic acid and heptafluorobutyric acid (Sigma); and *Firefly* luciferase reporter vector pGL3-basic and the dual luciferase expression system (Promega).

Cell Cultures
Bovine aortic ECs (BAECs), 8 to 12 passages, were purified and maintained in DMEM supplemented with 10% FBS and antibiotics as previously described.$^1$ Human coronary artery ECs (HCAECs, Clonetics), 4 to 7 passages, were maintained in EGM-MV medium supplemented with 20% FBS and antibiotics. For experiments, all cultures of subconfluent BAECs and HCAECs were incubated with PBS (control), homocysteine, cysteine, or methionine in the presence of 10 µmol/L EHNA and 50 µmol/L adenosine according to the protocol previously described.$^2$

Inhibitors of Signal Transduction Pathways
To determine the involvement of major signal transduction pathways, cells were treated with pertussis toxin (PTX, a Gi protein inhibitor), PD-98059 (a specific MEK/MAPK inhibitor), HA-1004 (a cGMP- and cAMP-dependent PK inhibitor), Ro-31-8220 (a specific PKC inhibitor), and BAPTA-AM (an intracellular Ca$^{2+}$ chelator) before being exposed to
homocysteine. All agents were purchased from Calbiochem. Protocols for individual agents were determined on the basis of the maximal doses and durations tolerable by the cells; tolerability was defined as <5% reduction in DNA synthesis.

**DNA Synthesis, Cell Count, and ELISA**

DNA synthesis was quantified by measuring $^3$H-thymidine incorporation. Cells were counted with a hemacytometer and percentage of dead cells was determined by trypan blue uptake. Levels of FGF2 and VEGF were measured by ELISA using Quantikine kits (R&D Systems).

**Cell Viability MTT Assay**

BAECs ($5 \times 10^4$ cells/well) were dispensed into 24-well plates and incubated for 24–96 hours after addition of homocysteine or cysteine, and the index of EC viability was determined by colorimetric MTT (tetrazolium) assay. Absorbance was read at wavelength of 595 nm for viable cells using a microplate reader (Thermo Electron Corporation).

**Fluorescence-Activated Cell-Sorting Analysis**

BAECs ($2–5 \times 10^5$ cells) were distributed across 60-mm dishes and cultured to 70–80% confluence. After 24 hours of various treatments, cells were fixed in 70% ethanol at 4°C and stained with propidium iodide (50 µg/mL)/DNase-free RNase (2 units/mL) at room temperature for 1 hour. Cells were then subjected to fluorescence-activated cell sorting analysis of DNA content in a flow cytometer (BD Biosciences).

**TUNEL Assay**

Apoptosis was assessed by detection of DNA fragmentation using the *in situ* DeadEnd™
Fluorometric TUNEL System (Promega). Subconfluent BAECs were subjected to various treatment for 24 hours. Adherent cells were fixed in 4% paraformaldehyde and incubated with the reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein-12-dUTP for 1 hour at 37°C. Cell images were analyzed with a fluorescence microscope (Leica DMIRB).

**Real-Time PCR Analysis**

Total RNA (5 µg) in 20 µL reaction mix was reverse transcribed using M-MLV Reverse Transcriptase (Promega) and real-time PCR was performed using the iCycler iQ™ Real-Time PCR Detection System (BioRad) with SYBR green according to the manufacturer’s protocol. Reactions were performed in a total volume of 20 µL, including 10 µL of ABsolute™ QPCR SYBR® Green Fluorescein Mix (ABgene, USA), 1 µL of each primer at a 5 µmol/L concentration, and 1 µL of the previously reverse transcribed cDNA template. The cycling conditions were as follows: 15 minutes at 95°C, and 40 cycles of 10 seconds at 95°C followed by 45 seconds at 55°C. Fluorescence threshold value was calculated using the iCycle iQ system software. The levels of each gene were standardized to β-actin for each sample. PCR primers for FGF2, VEGF, FGFR1, VEGFR2 and β-actin are listed in Supplemental Table 1.

**Reporter Gene Assay**

FGF2 promoter reporter gene assay was performed using a dual luciferase expression system (Promega). Human FGF2 5′-flanking sequences were PCR-amplified from human genomic DNA using the primers listed in Supplemental Table 1. These PCR products were fused to the Firefly luciferase reporter vector pGL3-basic and sequenced completely. HCAEC grown to 80% confluence in a 12-well plastic dish were transfected with 0.75 µg pGL3-basic or
equimolar pGL3-FGF2 constructs by Superfect™ reagent (QIAGEN). Renilla luciferase expression vector phRL-TK (0.5 µg), whose activity is unaffected by homocysteine, was cotransfected as an internal control. Twenty-four hours after transfection, cells were treated with 100 µmol/L homocysteine for 24 hours before cell lysates were prepared for luciferase assay using Luciferin and a luminometer (Packard Harvester). Promoter activities were normalized to phRL-TK and expressed as fold increase over pGL3-basic.

Promoter Sequence Analysis

Human FGF2 5'-flanking sequences were retrieved from GenBank as described.6 The CpG islands of human FGF2 gene were analyzed by the “CpG Island Searcher” available at http://www.cpgislands.com.7,8 Transcription factor binding sites were analyzed using the TRANSFAC database available at http://www.gene-regulation.com/.9

Measurement of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH)

SAM and SAH concentrations were measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) according to the procedures previously described.10 Briefly, HCAECs (1×10⁶ cells/100-mm dish) were suspended in 500 µL of ice-cold aqueous mobile phase (4 mM ammonium acetate, 0.1% formic acid, 0.1% heptfluorobutyric acid, pH 2.5) containing 1 µM [³H]-SAM and sonicated immediately for 10 seconds on ice to produce a homogeneous solution. A 10-µL aliquot was retained for determination of protein concentration using the RC DC™ protein assay reagent (Bio-Rad, Hercules, CA). The remaining solution was heated at 80°C for 5 minutes to precipitate endogenous proteins, cooled immediately on ice for 2 minutes and centrifuged for 15 minutes at 12,000×g to remove any precipitate. Samples were analyzed immediately by LC–MS/MS (API 3000™ LC/MS/MS System, Applied Biosystems, Foster City, USA) at the Department of Medical
Genetics, National Taiwan University Hospital (Taipei, Taiwan). Data were acquired and processed using Analyst for Windows NT software.

**Bisulfite Genomic DNA Sequencing**

DNA methylation was investigated using HCAECs cultured to 80 to 90% confluence and subsequently subjected to different treatments followed by genomic DNA extraction according to standard procedures. The reaction of genomic DNA (2 µg) with bisulfite using EpiTect® bisulfite kit (QIAGEN, Valencia, CA) converted all unmethylated cytosine residues to uracil. Bisulfite-modified DNA was amplified with FGF2 specific primers listed in Supplemental Table 1. These primers were designed by using MethPrimer, an online program for designing primers for bisulfite-based methylation PCR (available at http://www.urogene.org/methprimer/index.html). The cycling conditions were as follows: 15 minutes at 95°C, and 40 cycles of 10 seconds at 95°C followed by 45 seconds at 55°C. DNA sequencing was performed for 10 plasmid clones from each treatment group.

**References**


Figure Legends to Supplemental Figures

**Supplemental Figure 1.** Reversible effects of Hcy on cell viability. Cells were incubated with PBS (●) or 100 (▼) μmol/L Hcy for 96 hours, or 100 μmol/L Hcy (○) for the first 24 hours followed by PBS through 96 hours. Cell viability was expressed as OD 595 mm. Values are mean±SEM (n=3). *P<0.05 vs PBS control. †P<0.05 vs Hcy treatment.

**Supplemental Figure 2.** Dose-dependent and time-dependent effects of Hcy on VEGF expression. A, BAECs. Left: Intracellular VEGF protein levels were assayed by ELISA in BAECs incubated with Hcy (25–500 μmol/L) for 24 hours. Right panel: BAECs were incubated with Hcy (100 μmol/L) for indicated time periods (24–96 hours). Cells treated with PBS for 96 hours were used as control. Values are mean±SEM (n=3). *P<0.05, **P<0.01 vs PBS control. B, HCAECs. Intracellular VEGF was assayed by ELISA in HCAECs incubated with Hcy (25–500 μmol/L) for 24 hours (left), or with Hcy 100 μmol/L for 24–96 hours (right). Values are mean±SEM (n=3). C, Measurement of mRNA by real-time PCR. Cells were incubated with PBS, 100 μmol/L and 500 μmol/L of Hcy for 24 hours, and total RNA was subjected to real-time PCR analysis with specific primers for FGF2, VEGF and VEGFR2. Values are mean±SEM (relative to PBS-treated samples) after normalization to β-actin (n=4). *P<0.05 vs PBS control.

**Supplemental Figure 3.** FGF2 promoter analysis. A, Schematic illustration of the FGF2 gene structure. The CpG sites and basal promoter is shown. B, CpG island sequence. Human FGF2 promoter contains 31 CpG sites as indicated. The primers used for methylation DNA sequencing are shown. The methylation status of the CpG dinucleotides in HCAECs treated with Hcy 100 or 500 μmol/L for 24 hours is illustrated. Unmethylated cytosines are indicated by stalks. Methylated cytosines are indicated by stalks with square heads.
Supplemental Figure 1
Supplemental Figure 2

A. VEGF protein in BAECs

B. VEGF protein in HCAECs

C. Real-Time PCR for mRNA
Supplemental Figure 3

A

- Hind III (-1001)
- +1 +660 (Exon 1/Intron 1 boundary)
- Hind III

-532

-100

-34

FGF2 basal promoter

5'...CCTCGGACGCGGCCGCCTCGGCTCGCGCACCCTGCTCCCGCGGCGGCCCGGGGCGGCTTCCCGCGCG...3'

(Bottom box: GC box)

B

CpG Primer 1S

-351 CTTCCTTGA TTGCAAATTG TCTACTTTGG GGTGGAAACG GCTTCTCCG
-301 TTTGAAAGCG TAGCCGGGAA AAAATGCGGG AGAAATTTGA GTTAAACTT
-251 TTTAAAGTG GATCCCGGCT GGGTGCCAG CAAAAGCCCC GCAGTGTTGA
-201 GAAAGCTAA AGTGGTTTGT GGGTGTTGGG GGGTGCGCG GGGTGACTT

CpG Primer 2S

-151 TTGGGGGATA AGGCCCCGGTG GAGCCCCGGG AATGCACAAAG CCCTGCCCG

CpG Primer 1AS

14 15 16 17 18 19 20 21

-101 GCTCCCGACG CGCCCGGGGG GCCCTCCGGCC TCTCCCCCGC CCCGGACTG

CpG Primer 2AS

-51 GGCCTGGCTCCGGCCAGAC TGAATGTCGCC CGCTTGGCCTG TTGTCGCCGA

Control

1 2 3 4 5 6 7 8 9 10 11 12 17 19 20 22 24 27 28 29 31

Hcy-100

Hcy-500

| : Unmethylated CpG site

| : Methylated CpG site
### Supplemental Table 1: Primer sequences used in real-time PCR, reporter gene constructs and CpG methylation studies

<table>
<thead>
<tr>
<th>Primer sequence (sense)</th>
<th>Primer sequence (antisense)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Real-time PCR</strong></td>
<td></td>
</tr>
<tr>
<td>FGF2</td>
<td>5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3' 5'-AGCAGCCGTGGCCATCTCTTGTAGTC-3'</td>
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<tr>
<td>VEGF</td>
<td>5'-GGACATCTTCCAGGAGTAT-3' 5'-TGCAACCGAGTCTGTTGT-3'</td>
</tr>
<tr>
<td>FGFR-1</td>
<td>5'-GGAGTGCTTACCGTTACTGCT-3' 5'-TCAGCTTTACAGACATTGAAAGAAGG-3'</td>
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<tr>
<td>VEGFR-2</td>
<td>5'-CTCGAATTAGTCAGAAGATGAGTCTCCACCAG-3' 5'-CGTGGATCCCAAAGGGGACGAGTCGTC-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-AACGCGAGAAGATGACCCAGATCATGTTT-3' 5'-AGCAGCCGTGGCCATCTCTTGTAGTC-3'</td>
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<tr>
<td><strong>Reporter gene construct</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>–968 to +43</td>
<td>5'-ACGCGTTATCCGATGCTCTGAAATGTC-3' 5'-CTCGAGCTACTGCTCGGGTTTCTGAGG-3'</td>
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<tr>
<td>–126 to +43</td>
<td>5'-ACGCGTACAGGAATGCTCAGCCTCTGC-3' 5'-CTCGAGCTACTGCTCGGGTTTCTGAGG-3'</td>
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<tr>
<td>–100 to +43</td>
<td>5'-TTACGCTGACTGAGTTCGAGGC-3' 5'-CTCGAGCTACTGCTCGGGTTTCTGAGG-3'</td>
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<tr>
<td>–34 to +43</td>
<td>5'-TTACGCTGACTGAGTTCGAGGC-3' 5'-CTCGAGCTACTGCTCGGGTTTCTGAGG-3'</td>
</tr>
<tr>
<td>+24 to +179</td>
<td>5'-ACGCGTATCCCCGTTCAGCAGGGCA-3' 5'-CTCGAGCTACTGCTCGGGTTTCTGAGG-3'</td>
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<tr>
<td><strong>DNA methylation</strong></td>
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<tr>
<td>CpG Primer 1</td>
<td>5'-TTGGATAGTTCATTATTTATTTATTTG-3' 5'-AACTTTAACATCCCTAAACTCCAC-3'</td>
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<td>CpG Primer 2</td>
<td>5'-TTGGATAGTTCATTATTTATTTATTTG-3' 5'-AACTTTAACATCCCTAAACTCCAC-3'</td>
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</tbody>
</table>

<sup>a</sup> Restriction sites added for cloning: *Mlu*<sup>-1</sup> (underlined) and *Xho*<sup>-1</sup> (double underlined).
Supplemental Table 2: Opposing effects of homocysteine (Hcy) and FGF2 on cell cycle transitions.

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>60.87 ± 4.07</td>
<td>25.76 ± 3.29</td>
<td>13.37 ± 1.48</td>
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<tr>
<td>Hcy</td>
<td>76.30 ± 3.01*</td>
<td>10.90 ± 5.10*</td>
<td>12.80 ± 2.31</td>
</tr>
<tr>
<td>Hcy + FGF2</td>
<td>64.30 ± 6.93</td>
<td>21.25 ± 1.06</td>
<td>14.45 ± 5.87</td>
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<tr>
<td>Hcy + VEGF</td>
<td>72.70 ± 4.80*</td>
<td>13.45 ± 3.75*</td>
<td>13.85 ± 1.06</td>
</tr>
</tbody>
</table>

BAECs were incubated with 100 µmol/L Hcy in the presence or absence of 50 ng/mL FGF2 or 50 ng/mL VEGF for 24 hours before fluorescence-activated cell sorter analysis. Distributions of the cells in the G1, S, and G2/M phases are tabulated. Values are mean±SEM (n=3). *P<0.05 vs PBS control.