Cystathionine-β-synthase gene transfer and 3-deazaadenosine ameliorate inflammatory response in endothelial cells

Utpal Sen, Neetu Tyagi, Munish Kumar, Karni S. Moshal, Walter E. Rodriguez, and Suresh C. Tyagi

Department of Physiology and Biophysics, University of Louisville School of Medicine, Louisville, Kentucky

Submitted 21 May 2007; accepted in final form 10 September 2007

Sen U, Tyagi N, Kumar M, Moshal KS, Rodriguez WE, Tyagi SC. Cystathionine-β-synthase gene transfer and 3-deazaadenosine ameliorate inflammatory response in endothelial cells. Am J Physiol Cell Physiol 293: C1779–C1787, 2007. First published September 13, 2007; doi:10.1152/ajpcell.00207.2007.—Although elevated levels of homocysteine (Hcy) known as hyperhomocysteinemia (HHcy) are associated with increased inflammation and vascular remodeling, the mechanism of Hcy-mediated inflammation and vascular remodeling is unclear. The matrix metalloproteinases (MMPs) and adhesion molecules play an important role in vascular remodeling. We hypothesized that HHcy induces inflammation by increasing adhesion molecules and matrix protein expression. Endothelial cells were supplemented with high methionine, and Hcy accumulation was measured by HPLC. Nitric oxide (NO) bioavailability was detected by a NO probe. The protein expression was measured by Western blot analysis. MMP-9 activity was detected by gelatin-gel zymography. We demonstrated that methionine supplement promoted upregulation of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) through increased Hcy accumulation. In addition, increased synthesis of collagen type-1 was also observed. MMP-9 gene expression and protein activity were increased in methionine supplement groups. 3-Deazaadenosine (DZA), an adenosine analogue, prevented high methionine-induced ICAM-1 and VCAM-1 expression and collagen type-1 synthesis. Transfection of endothelial cells with cystathionine-β-synthase (CBS) gene construct, which converts Hcy to cystathionine, reduced Hcy accumulation in high methionine-fed cells. CBS gene transfection reduced the inflammatory response, as evident by attenuated ICAM-1 and VCAM-1 expression. Furthermore, collagen type-1 expression and MMP-9 activity were dramatically attenuated with CBS gene transfection. These results suggested that methionine supplement increased Hcy accumulation, which was associated with inflammatory response and matrix remodeling such as collagen type-1 synthesis and MMP-9 activity. However, in vitro DZA and CBS gene therapy successfully treated the HHcy-induced inflammatory reaction in the methionine metabolism pathway.

extracellular matrix; matrix metalloproteinase-9; intercellular cell adhesion molecule-1; vascular cell adhesion molecule-1; collagen type-1; hyperhomocysteinemia

ENDOTHELIAL CELLS comprise an important cellular component of the normal arterial wall. This single-cell lining of the circulatory system maintains normal vascular physiology and dysfunction. The endothelium is a critical factor in the pathogenesis of vascular diseases such as atherosclerosis/arteriostenosis. This is a chronic inflammatory disorder and experimental evidence implicates sustained elevation of cell adhesion molecules such as intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) during this process (30). Hyperhomocysteinemia (HHcy), a dysregulated product of methionine metabolism, has now been recognized as a pathophysiological stimulus of arterial endothelial dysfunction that is specifically relevant to atherogenesis/arteriogenesis and considered an independent vascular risk factor. HHcy has been shown to increase expression of ICAM-1 (5) and VCAM-1 (11) in rats and mice, respectively. Among the atherogenic plaque protein, collagen constitutes up to 60% and type-1 collagen comprises approximately two-thirds of the total collagen (26). Too much collagen in the arterial wall leads to arterial stenosis (26). However, during methionine supplement, the inflammatory reaction and increased synthesis of collagen as a result of excessive Hcy accumulation is not fully defined.

Matrix metalloproteinases (MMPs) are Zn-containing endopeptidases that are actively involved in extracellular matrix (ECM) remodeling (21), and studies have demonstrated that HHcy induces vascular remodeling through activation of these peptidases (7). In the MMPs family, MMP-9 has received particular attention in analysis of vascular remodeling following tissue injury and inflammation (38). Recently MMP-9 has been shown to impair both collagen assembly and compaction (14). Interstitial collagen remodeling by MMPs leads to increase ECM formation (23) and increase in MMP-9 activity. These events have more pronounced effect during early and late phases of cardiovascular remodeling (34).

The 3-deazaadenosine (DZA) has been used for a long time as an anti-inflammatory drug, and its mechanism of action is generally thought to be mediated through the inhibition of methylation reactions (36). DZA is a potent inhibitor of S-adenosylhomocysteine hydrolase (SAHH), an enzyme that converts S-adenosylhomocysteine to Hcy during methionine metabolism, thereby reducing Hcy accumulation (1). Hcy metabolism is linked to transsulfuration pathway that converts intermediate product of methionine cycle Hcy to cysteine (25). The enzyme cystathionine-β-synthase (CBS) controls the conversion of Hcy to cystathionine under normal conditions and deficiency leads to severe HHcy. Vascular endothelial cells lacking the CBS gene are the prime target of Hcy toxicity leading to initiation and progression ECM remodeling during methionine supplement. However, the precise mechanism by which HHcy initiates this process is incompletely defined.

We hypothesize that high methionine-induced HHcy causes an inflammatory reaction that involves upregulation of adhesion molecules expression, increases accumulation of ECM components such as collagen type-1, and activates MMP-9, which takes an active part in ECM remodeling.
MATERIALS AND METHODS

Cell culture. Mouse aortic endothelial cells (MAEC) were a generous gift from Kathleen Bove Stratton, VA Medical Center, Albany, NY. Cells were maintained in DMEM/F-12 50/50 (Cellgro, Mediatech, Herndon, VA) as described earlier (29) in a humid chamber at 37°C in an atmosphere of 5% CO2 and 95% air. Confluent T-25 flasks were trypsinized (0.25% trypsin-2.21 mM EDTA in Hanks’ balanced salt solution without sodium bicarbonate, calcium, and magnesium) (Cellgro, Mediatech, Herndon, VA) to detach cells, resuspended in fresh complete culture medium, plated onto a 12-well cell culture plate, and allowed to grow about 80% confluence before experiments.

Antibodies and reagents. Antibodies to ICAM-1, VCAM-1, endothelial nitric oxide (eNOS), MMP-9, collagen type-1, and horseradish peroxidase-linked anti-mouse, anti-rabbit, and anti-goat IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CBS antibody was from Novus Biologicals (Littleton, CO). Mouse monoclonal antibody against β-actin, DZA, Nω-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), and other analytical reagents were from Sigma (St. Louis, MO). Polyvinylidene difluoride membrane was from Bio-Rad (Hercules, CA).

HPLC apparatus. High-pressure liquid chromatography (HPLC) analyses were performed in a Shimadzu Class-VP 5.0 chromatograph (Shimadzu) equipped with LC-10ADvp pump, SIL-10ADvp autoinjector, CTO-10Avp column oven, and SPD-10Avp detector. We used a premier C18 amide 5-µm 150 × 2.1 mm column to detect Hcy, and during the sample run, the oven temperature was constantly maintained at 37°C.

Chromatography. The chromatographic conditions were maintained as described elsewhere (18). Briefly, 0.1 M monochloroacetic acid and 1.8 mM octylsulfate mixed together, adjusted to pH 3.2, and was used as mobile phase. Before being used, this solvent was filtered through a Millipore filter (0.45 µm) and degassed under vacuum. The isocratic solvent was pumped and circulated through the column at a constant flow of 0.8 ml/min. Samples were injected through autoinjector, and an injection volume of 20 µl was used. During HPLC analysis, Hcy in culture supernatants were identified according to their retention times and co-chromatography with standards.

Sample preparation. Culture supernatants were collected and centrifuged to remove cell debris. To determine Hcy in the supernatants, 200 µl of supernatant were diluted with 100 µl of water and then 300 µl of 9 M urea (pH 9.0) were added. The 50 µl of n-amylnalcohol was added to the solution as an antifoaming agent. Reduction of disulfides and cleavage of the protein-bound, sulfur-containing amino acids were performed by the addition of 50 µl of NaBH4 solution (10%, wt/vol) in 0.1 N NaOH. To perform the reaction, samples were incubated in a water bath at 50°C for 30 min. Samples were cooled down at room temperature and the reaction was stopped by the addition of 500 µl of 20% trichloroacetic acid. The proteins were separated by centrifugation for 4 min at 12,000 g, and supernatants were filtered using a 0.45-µm Millipore filter.

Nitric oxide. The NO was measured with WPI potentiometer, interfaced with a PC, using an NO-specific probe.

RT-PCR. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed according to the previously described procedure (29). The eNOS primers for PCR were 350-bp product sense (5′-TTCCGGCTGCCACCTGATC-3′) and antisense (5′-AACATAT-GTCCTTGCTCAA-3′). The PCR thermal cycle was 94°C for 5 min, then 35 cycles of 94°C for 50 s, 60°C for 50 s, and 72°C for 1 min, and finally 72°C for 5 min were performed. Primers and PCR thermal cycle for GAPDH were the same as used earlier (29). All the primers were obtained from Invitrogen (Carlsbad, CA).

Fig. 1. Chromatograph of homocysteine (Hcy) from cell culture medium. A: control with 1× methionine (17.2 mg/l); B: cells treated with 10× methionine; C: cells treated with 10× methionine + 3-deazaadenosine (DZA, 100 µM). D: dose dependence for the levels of Hcy over a range of DZA (0–100 µM). See MATERIALS AND METHODS for details of chromatographic condition. #Significant difference (P < 0.05) compared with control (n = 4).
**Expression vectors and CBS transfections.** The CBS cDNA was kindly provided by Dr. Jeffrey Taub (33). The CBS cDNA and green fluorescent protein (GFP) cDNA (0.75 kilobase pairs) were subcloned into the plasmid pcDNA3.1. The pcDNA3.1/GFP and pcDNA3.1/CBS plasmids were purified using QIAGEN Plasmid Miniprep Kit (Chatsworth, CA) according to the manufacturer’s instructions. MAECs were grown to 50–60% confluence in OPTI MEM medium and transfected with pcDNA3.1/GFP and pcDNA3.1/CBS plasmids (0.2 μg DNA/cm²) using Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer’s guidelines. Forty-eight hours after transfection, the percent transfection efficiency was determined by examining pcDNA3.1/GFP-transfected cells for GFP fluorescence using laser confocal microscope (Fluo View 1000, Olympus). CBS expression was determined by Western blot analysis using anti-CBS antibody as well as indirectly by NO production and HPLC analysis of Hcy (data not shown in this communication) in high methionine-treated culture medium.

**Immunoblot analysis.** Cells were lysed in ice-cold modified RIPA lysis buffer containing appropriate proteinase inhibitor as described previously (29). Protein content of the lysate was determined using Bradford protein-assay reagent (Bio-Rad). Protein samples were mixed with 1:1 vol/vol ratio with 2× sample loading buffer [800 μl glycerol; 1 ml Tris·HCl 0.5 mM (pH 6.8); 1.6 ml 10% (wt/vol) SDS; 400 μl 2-mercaptoethanol; 400 μl 0.05% (wt/vol) bromophenol blue], boiled at 95–100°C for 5 min. Samples were cooled down to room temperature and then centrifuged to precipitate cell debris. Equal amount of protein (15 μg) for each sample was resolved by 10% SDS-PAGE. Protein in the gel was then electrophoretically transferred to polyvinylidene difluoride membrane. Transferred protein was blocked with 5% nonfat dry milk in TBS-T (50 mM Tris·HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 h at room temperature followed by incubation with appropriate primary antibodies in blocking solution for another 1 h. After three washes with TBS-T, membranes were incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase for 1 h. Four more 10-min washes were performed, and Amersham ECL Plus substrate (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was applied to the blots for 5 min. The blots were developed using X-ray film (RPI, Mount Prospect, IL) with a Kodak 2000A developer (Eastman Kodak, Rochester, NY). Image analysis was performed using UMAX PowrLock II (Taiwan, Republic of China).

**Zymography.** MMP-9 activity in cultured endothelial cells was measured using gelatin-gel zymography as described previously (19). Briefly, 40-fold concentrated conditioned culture medium was electrophoretically resolved by 7.5% SDS-PAGE containing 2 mg/ml gelatin as a substrate. At the end, gel was incubated in renaturation buffer (2.5% Triton X-100) for 30 min to remove SDS, rinsed in distilled water, and then incubated overnight at 37°C in water bath in activation buffer (50 mM Tris·HCl, pH 7.4, and 5 mM CaCl₂). Gel was stained using 0.5% Coomassie blue R-250 for 1 h. MMP activity was detected as a white band on a dark blue background and quantitated densitometrically using Un-Scan-It software (Silk Scientific, Orem, UT).

**Statistical analysis.** Results were expressed as means ± SE; n = 4 experiments/group unless mentioned otherwise. Differences between groups were tested using two-way ANOVA with repeated measures. Comparisons within groups were made with the use of one-way ANOVA followed by Scheffé’s post hoc test. Comparisons between groups were made with the use of Student’s independent t-test, with P < 0.05 considered statistically significant.

**RESULTS**

**Role of DZA in Hcy accumulation.** Cell culture medium was collected after 48 h from control (1× methionine; 17.2 μg/ml), 10× methionine-, and 10× methionine + DZA (100 μM)-treated cells. The medium was analyzed by HPLC. There was increased accumulation of Hcy in the high methionine-treated group compared with that in the control (Fig. 1, A and B). The Hcy levels were 3.1 ± 0.5, 33.55 ± 4.3, and 5.3 ± 0.62 μM in the culture supernatant of control, high methionine-, and high methionine + DZA-treated cells, respectively. These levels were similar and largely comparable with in vivo studies (4, 23). The elution time for the Hcy was about 1.35 min as detected with standard (data not shown). The increase in Hcy accumulation in the high-methionine group was attenuated by DZA treatment, which suggested that DZA was a potent inhibitor of Hcy formation (Fig. 1C). To determine the dose dependence, we investigated whether various concentrations of DZA (0–100 μM) had an effect on high methionine (10×)-
supplemented cells to lower Hcy accumulation. Figure 1D showed a dose-dependent inhibition of Hcy concentration in the collected supernatant. Although we found a trend of an Hcy-lowering effect at doses 10 and 25 μM/9262 M, a significant difference was observed at doses 50 and 100 μM of DZA. For the subsequent studies we used 100 μM DZA dose. This dose had also been used for in vitro studies by several other groups (13, 15).

**Endothelial cells ability to generate NO.** The acetylcholine-mediated NO formation had been used to assess endothelial cell integrity (9). The 10−5 M of acetylcholine was the concentration that caused maximal vasodilation through an endothelium-dependent NO pathway and had widely been used in vitro by our group and others (3, 8, 10, 31). Therefore, NO generation in response to an optimal dose of acetylcholine (10−5 M) was used to assess endothelial viability in the presence and absence of high levels of methionine with or without DZA (100 μM). We observed that endothelial cells capability to generate NO in the presence of Hcy accumulation, in cells fed high levels of methionine, was attenuated (Fig. 2). Interestingly, when cells were given high methionine together with DZA, the production of NO following acetylcholine administration returned toward normal. This result suggested that DZA was an effective tool to normalize endothelial integrity under high-methionine conditions.

**High methionine dose dependently inhibits eNOS activity.** To determine whether methionine decreased NO in part by decreasing eNOS mRNA and protein levels, the effect of various concentrations of methionine on the eNOS gene and protein expression were measured. Methionine supplement attenuated eNOS mRNA expression at 10× (Fig. 3A), whereas the protein levels were effected at 5× methionine (Fig. 3B). The purpose of the experiments in Fig. 3 was to determine the optimal effective dose of methionine in the decrease of mRNA and protein expression of eNOS, whereas the focus of experiments in Fig. 4 was to mitigate the induction of inflammatory markers (ICAM-1 and VCAM-1) by optimal effective methionine dose by DZA. Therefore, we tested DZA in Fig. 4.

**High methionine induces adhesion molecules.** The adhesion molecules ICAM-1 and VCAM-1 were increased with high-methionine treatment (Fig. 4). When high methionine was given in combination with DZA (100 μM), the expression of...
adhesion molecules were mitigated; however, DZA alone had no effect on adhesion molecule expression (Fig. 4). These results provided mechanistic evidence that in endothelial cells methionine supplement caused accumulation of Hcy, which in turn induced endothelial cell injury. This injury led to the induction of adhesion molecules, which in vivo was recognized as a marker of inflammation (22). DZA, however, prevented Hcy accumulation thereby inhibited adhesion molecules expression and inflammation.

**Methionine supplement modulates MMP-9.** The high methionine induced about three-fold MMP-9 protein expression (Fig. 5A, left), whereas DZA treatment, together with high methionine, regressed this induction in MMP-9 levels. The increase in MMP-9 expression was due, in part, to the inhibition of eNOS activity, which resulted in the decrease in bioavailability of NO. l-NAME (eNOS inhibitor) increased MMP-9 expression 3.5-fold, and SNP (NO-donor, 10^{-5} M) when used along with high-methionine MMP-9 was almost normal level (Fig. 5A, right). These results demonstrated that eNOS-derived NO had a major role in regulating MMP-9 expression and activity, and high methionine affected MMP-9 expression and activity through modulating NO generation (Fig. 5B).

**Collagen synthesis in response to high methionine.** Collagen is an integral part of vascular remodeling, and type 1 collagen deposition is observed in fibrotic atherosclerotic remodeled plaque (26). Our results showed that high methionine increased synthesis and release of collagen type-1 in the endothelial culture (Fig. 5C), whereas DZA attenuated this synthesis and release. In addition, the increase in collagen type-1 synthesis was observed with l-NAME, which was normalized by SNP in the methionine-treated group. These results confirmed the role of eNOS-derived NO in the synthesis and accumulation of collagen type-1 and that high methionine had a role in NO generation through Hcy accumulation.

**CBS transfection reverses endothelial dysfunction.** To better understand the relationship of CBS gene expression and Hcy accumulation thereby inhibited adhesion molecules expression, and inflammation.

**DISCUSSION**

Methionine supplement developed HHcy that induced the increase in expression of adhesion molecules ICAM-1, VCAM-1, and ECM protein collagen type-1. In in vivo conditions, these adhesion molecules lead to inflammation, and together with ECM remodeling, play a critical role in the development and progression of atherosclerosis/arteriosclerosis (11, 22). MMP-9, required for collagen assembly and compaction, was increased by Hcy. The inhibition of methylhylation process by DZA and CBS transfection reduced the Hcy accumulation in high methionine-fed cells. DZA is a potent inhibitor of SAHH, which converts S-adenosylhomocysteine to Hcy, whereas CBS gene is a key enzyme of transsulfuration pathway that converts Hcy to cystathionine (Fig. 8). DZA inhibited the production of Hcy, whereas CBS cleared Hcy. Reduced Hcy accumulation attenuated adhesion molecules expression, MMP-9 expression, and activity as well as collagen type-1 synthesis, which may inhibit atherosclerosis/arteriosclerosis processes induced by Hcy.

![Image](http://ajpcell.physiology.org/)

Fig. 5. Methionine modulates matrix metalloprotei-nase (MMP-9) (A and B) and collagen type-1 (C) synthesis. Cells were grown onto cell culture plate, supplemented with high methionine, and treated with or without DZA (100 μM), eNOS inhibitor (N^o-nitro-l-arginine methyl ester, l-NAME), or NO donor (SNP) for 2 days. In each experiment appropriate controls were taken. Cells processed for Western blot analysis and culture supernatant for zymography as described in MATERIALS AND METHODS. A: top: Western blot of MMP-9. Bottom: MMP-9 expression over β-actin loading control (**P < 0.05, compared with control). B: top: activity of MMP-9 in gelatin zymography. Bottom: Fold changes of MMP-9 activity over control (#P < 0.05, compared with control). Values are means ± SE; n = 4 experiments in each group. Note that both DZA and SNP inhibit MMP-9 expression and activity in methionine-treated cells, whereas methionine and l-NAME induces MMP-9 expression and activity. C: conditioned cultures medium concentrated 20× and Western blot analysis performed with anti-collagen type-1 antibody.
Arteriosclerosis is the vascular event of endothelial cell injury characterized by inflammatory reactions, degenerative changes, and accumulation of ECM (27). One of the early events of arteriosclerosis/atherosclerosis is the decreased bioavailability of NO in endothelial cells, resulting in expression of pro-inflammatory molecules such as ICAM-1 and VCAM-1 (36). The increased proportion of collagen type 1 is the characteristic of atherosclerotic intimal plaque (26), MMP-9 plays an important role in chronic airway inflammation (17). However, very little is known about the inflammatory process and the development of arteriosclerosis/atherosclerosis during HHcy.

Endothelium plays a variety of key roles, including endothelium-dependent vasorelaxation mediated by NO. One form of endothelial injury is characterized by impaired endothelium-dependent NO availability, which contributes to many cardiovascular diseases, including arterial hypertension, hypercholesterolemia, and endothelial myocyte uncoupling associated with arterial remodeling (2, 23, 28). High methionine causes accumulation of Hcy, and increased Hcy is associated with decreased NO bioavailability in cultured endothelial cells (32, 35). Since NO derived from the endothelium inhibits platelet aggregation and leukocyte adhesion to the vascular wall, decreased bioavailability of NO in Hcy-induced oxidative stress causes oxidative inactivation of NO, which leads to inflammatory reactions resulting in initiation and acceleration of arteriosclerosis/atherosclerosis (35, 37). In accordance with these studies, our in vitro experiments show that methionine supplementation causes excessive accumulation of Hcy (Fig. 1), and increase in Hcy accumulation leads to decrease bioavailability of endothelium-derived NO in response to physiological stimuli such as acetylcholine (Fig. 2). In addition, high methionine in cultured endothelial cells dose-dependently decreases the eNOS (the enzyme produces NO) gene and protein expression as revealed by RT-PCR and immunoblot analyses (Fig. 3).

Experimental evidence implicates arteriosclerosis/atherosclerosis is a chronic inflammatory disorder (27) associated with sustained elevation of cell adhesion molecules such as ICAM-1 and VCAM-1, through which monocyte adheres and subsequently migrates into the vessel wall (30). Moderately elevated Hcy has been found to upregulate VCAM-1 expression (30) as well as ICAM-1 RNA transcription and cell surface expression in endothelial cells (24). These two adhesion molecules play an important role during the development of atherosclerosis (36). Moreover, recent studies showed that DZA inhibits formation of atherosclerotic lesions through inhibition of endothelial expression of ICAM-1 and VCAM-1 in the atherosclerotic mice model (16, 36). This present study demonstrates strong evidence that high methionine upregulates pro-inflammatory molecules ICAM-1 and VCAM-1 in endothelial cells (Fig. 4) through an accumulation of Hcy and serves as a mediator of atherosclerotic process. The anti-atherogenic effect of DZA is associated with a marked decrease in Hcy level in the culture supernatant and a concomitant decrease of endothelial expression of ICAM-1 and VCAM-1 in high methionine-fed cells in vitro. These findings support the hypothesis that DZA is a strong and effective drug in normalization of Hcy- and Hcy-associated inflammation and development of arteriosclerosis/atherosclerosis.

Our previous study using an in vivo animal model suggested impaired arterial function in HHcy. This was the consequence of ECM accumulation due to MMP activation (23). The collagenolytic activity and MMP-9 gene expression were dramatically increased and were correlated with aortic ECM deposition (23). It has been reported that collagen increased in atherosclerosis, and type-1 collagen was vital for fatty streak and plaque formation during atherosclerosis (6). Synthesis, secretion, and degradation of type-1 collagen is a dynamic equilibrium process, and when synthesis and secretion of collagen exceed resolution, collagen protein deposits in the arterial wall and actively participates in the initiation and progression of atherosclerosis (20). In accordance with our previous in vivo study and others, our present in vitro study showed increase in MMP-9 activity and collagen type-1 deposition in methionine-supplemented cells that were attenuated by DZA treatment (Fig. 5). Accumulated Hcy was associated with matrix remodeling as evident by an increase in MMP-9 activity and collagen-I deposition. Importantly, our data showed that DZA was the drug that can effectively regulate this process through interfering with Hcy accumulation and inflammation.

Hcy is produced from methionine through the intermediates $S$-adenosyl methionine (SAM) and $S$-adenosyl homocysteine (SAH). CBS is the gene that converts homocysteine to cysteine.
thionine and thereby reduces Hcy accumulation in the body (Fig. 8). Endothelial cells appear to lack the CBS gene, and therefore methionine supplement dramatically affects Hcy clearance resulting in increase proportion of Hcy in endothelial cells (3). This particular effect is more acute in CBS-deficient individuals where increased total plasma Hcy leads to increased uptake of Hcy in endothelial cells (4), causing a risk of cardiovascular events (12). In an effort to reduce Hcy accumulation in methionine-supplemented cultured endothelial cells, we transfected endothelial cells with CBS gene construct. Our results suggested that appearance of Hcy in the culture supernatant was significantly low in comparison with control transfection with methionine supplement (data not shown). More interestingly, the decrease production of NO was normalized in CBS-transfected cells (Fig. 6), which was comparable with DZA treatment with high-methionine supplement (Fig. 2). In addition, methionine supplement-induced upregulation of ICAM-1 and VCAM-1 was dramatically attenuated in CBS-transfected cells (Fig. 7, A and B). MMP-9 expression and activity along with collagen type-1 synthesis were also regressed as well (Fig. 7C).

In summary, the present study provides strong mechanistic evidence that high doses of methionine impaired normal endothelium-dependent NO production through accumulation of Hcy. Excessive Hcy accumulation was associated with increased expression of pro-inflammatory cellular adhesion molecules such as ICAM-1 and VCAM-1 that were known to

Fig. 7. CBS limits inflammatory reaction and ECM remodeling. Endothelial cell were transfected with CBS cDNA subcloned into the plasmid pcDNA3.1 as described in MATERIALS AND METHODS. Methionine-supplemented cells (48 h) were processed for Western blot analysis. Conditioned culture medium was used for gelatin-gel zymography and collagen analysis. A: CBS transfection showing increased expression of CBS protein in the cells (top). Bottom: CBS limits ICAM-1 and VCAM-1 expression. B: densitometric analyses of ICAM-1 (top) and VCAM-1 (bottom) against β-actin. C: similar sets of experiments cell lysates were processed for MMP-9 expression, and serum-deprived culture medium used for MMP-9 activity and collagen expression. D-F: fold change (×control) of MMP-9 protein expression, enzyme activity, and collagen type-1 expression, respectively. #Significant changes over control (P < 0.05, n = 4).

Fig. 8. Schematic of Hcy-induced inflammation and ECM remodeling. Hcy produced from methionine through intermediate conversion of S-adenosylmethionine (SAM) and S-adenosyl-homocysteine (SAH). Endothelial cells lack CBS activity, therefore, they accumulated Hcy in methionine supplement medium. Increased Hcy induces endothelial cell injury and promoted upregulation of inflammatory molecules such as ICAM-1 and VCAM-1. Increase synthesis of collagen type 1 and MMP-9 activity is associated with HHcy-related inflammatory reaction and matrix remodeling.
promote arteriosclerosis/atherosclerosis through leukocyte adherence and cellular transmigration. Our data also showed that high-methionine supplement accelerated vascular remodeling through MMP-9 activation and increased in synthesis of collagen type-1. In the in vivo situation these events were strongly related to arteriosclerosis/atherosclerosis. This study indicated that DZA or CBS gene therapy could effectively be used to reduce total Hcy and consequent arteriosclerosis/atherosclerosis. However, further studies are necessary to reveal more mechanistic detail in the events of DZA treatment and CBS gene therapy on reducing HHcy, which is specifically relevant to the pathogenesis of inflammation and matrix remodeling leading to arteriosclerosis/atherosclerosis.

One of the limitations of this study was that although we used 100 μM of DZA in vitro conditions, it remained to be validated whether this dose was physiologically or pharmacologically relevant in in vivo animal treatment. DZA was not administered to methionine-fed mice; rather, static studies in endothelial cells were performed. Only in the in vivo setting might be the integration of distinct responses (i.e., smooth muscle cells and the immune system—important contributors to atherosclerosis) be fully studied and clarified.

GRANTS
A part of this study was supported by National Institutes of Health Grants HL-71010, NS-51568, and HL-88012.

REFERENCES


