Effects of Folic Acid and Magnesium on the Production of Homocysteine-Induced Extracellular Matrix Metalloproteinase-2 in Cultured Rat Vascular Smooth Muscle Cells

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Background  Hyperhomocysteinemia is an independent risk factor of coronary artery disease, but some studies have shown that patients with hyperhomocysteinemia are not prone to atherosclerosis. The aim of this study was to test whether homocysteine increases the production of matrix metalloproteinase-2 (MMP-2) and if extracellular additional magnesium and folic acid alters MMP-2 secretion.

Methods and Results  Gelatin zymography and western blotting were used to investigate the effects of different homocysteine levels (0–5,000 μmol/L) on MMP-2 production, and the effects of different folic acid concentrations (0–10 μmol/L) and magnesium concentrations (0–3.0 mmol/L) on homocysteine-induced MMP-2 in cultured rat vascular smooth muscle cells. Furthermore, the changes in MMP-2 were compared under various treatments for 24 h, 48 h and 72 h. Homocysteine (50–1,000 μmol/L) increased the production of MMP-2 significantly in a dose-dependent manner and at a high level (5,000 μmol/L) reduced the production of MMP-2. Increased production of MMP-2 induced by homocysteine was reduced by additional extracellular folic acid in a dose-dependent manner. Magnesium also reduced the increase of MMP-2 production induced by homocysteine. Production of MMP-2 under various treatments for 72 h increased more than during 24 or 48 h.

Conclusions  Homocysteine (50–1,000 μmol/L) significantly increased the production of MMP-2 in a dose-dependent manner. Added extracellular folic acid and magnesium decreased the homocysteine-induced MMP-2 secretion. These data suggest a beneficial effect of folic acid and magnesium on the pathogenesis of coronary artery disease. (Circ J 2006; 70: 141–146)

Key Words: Atherosclerosis; Folic acid; Homocysteine; Magnesium; Matrix metalloproteinase; Smooth muscle cells
duced MMP-2 in cultured VSMCs has not been studied to our knowledge. The aim of this study was to test whether additional extracellular magnesium and folic acid would alter MMP-2 secretion.

Methods

Materials

The chemicals used in this study were obtained from the following sources: Dulbecco’s modified Eagle’s medium (DMEM) without magnesium was obtained from GIBCO (Grand Island, NY, USA); fetal calf serum (FCS) was purchased from Filtron Pty Ltd (Tokyo, Japan); D-L-homocysteine was purchased from Nacalai Tesque Inc (Kyoto, Japan); folic acid was supplied by Sigma-Aldrich Co (St Louis, MO, USA); rabbit anti-MMP-2 antibody (NeoMarkers antibody line, Ab-7, RB-1537-PO) was obtained from Lab Vision Co (Westinghouse Drive, Fremont, CA, USA). All other chemicals were of reagent grade or the highest grade commercially available.

Preparation of VSMCs

Rat aortic VSMCs were isolated by enzymatic digestion from the thoracic aortas of 6-week-old male Sprague-Dawley rats (Charles River Japan, Kanagawa, Japan) as described previously. All surgical interventions and anesthesia were conducted in conformity with institutional guidelines and in compliance with international laws and policies (EEC Council Directive 86/609, OIL 358, December 1987; Guide for the care and use of laboratory animals, NIH publication No. 85-23, 1985). The cells were cultured in DMEM supplemented with 10% FCS at 37°C in a humidified 5% CO2 to 95% air atmosphere. At confluence, cells displayed a ‘hill and valley’ growth pattern and abundant myofilaments in their cytoplasm. They were identified as VSMCs by immunocytochemistry using HHF35, a monoclonal antibody that recognizes muscle-specific actin. All SMC cultures used in this study were between passages 4 and 7. At the subconfluent stage, the culture medium was replaced with serum-free medium and then the cells were exposed to various treatments.

Preparation of Culture Medium

Homocysteine was added to DMEM without magnesium to a final homocysteine concentration of 0–5,000 μmol/L; folic acid was added to DMEM without magnesium to a final concentration of 0–10 μmol/L; magnesium sulfate (MgSO4) or magnesium chloride was added to DMEM without magnesium to a final magnesium concentration of 0–3.0 mmol/L before use. The level of 6.25–12.5 mmol/L homocysteine is physiological and >25 mmol/L is pathological. The level of 0–0.5 mmol/L magnesium corresponds to the lowest physiological level of this ion that can be measured in human serum, 1.0 mmol/L to the physiological level, and 3.0 mmol/L to the highest level reached by therapeutic supplementation of magnesium in patients with arrhythmia or preeclampsia. All folic acid and magne-
sium treatments were sustained in the 500\(\mu\)mol/L homocysteine-treated media for 72 h.

**Analysis of Gelatinase Production**

After various treatments for 24 h, 48 h and 72 h, medium samples were harvested, centrifuged at 2,000 G for 10 min and normalized for cell protein content using a Bio-Rad assay. The samples were applied without reduction to a 7.5% polyacrylamide slab gel impregnated with 1 mg/ml gelatin. After electrophoresis, the gel was washed at room temperature for 30 min in washing buffer (50 mmol/L Tris–Cl, pH 7.5, 15 mmol/L CaCl\(_2\), 1\(\mu\)mol/L ZnCl\(_2\), and 2.5% Triton X-100), then incubated overnight at 37°C with shaking in the same buffer but containing 1% rather than 2.5% Triton X-100. The gel was stained with a solution of 0.1% Coomassie brilliant blue R-250. Clear zones against the blue background indicated the presence of gelatinase. To quantify the amount of gelatinase production, the stained zymograms were scanned on a densitograph (ATTO, Tokyo, Japan).

**Western Blotting**

After various treatments for 24 h, 48 h and 72 h, medium samples were harvested from the cells, using the protease inhibitors phenylmethane sulfonyl fluoride (0.1 mmol/L) and leupeptin (10\(\mu\)g/ml), centrifuged at 2,000 G for 10 min and separated by electrophoresis on 7.5% sodium dodecyl sulfate polyacrylamide gels followed by transfer onto polyvinylidene difluoride membranes (Immobilon P, Millipore, 0.22\(\mu\)m pore size). The membranes were blocked in 5% skim milk in phosphate-buffered saline containing 0.1% Tween 20 at room temperature for 1 h, and probed with anti-MMP-2 monoclonal antibody overnight. After washing 3 times with phosphate-buffered saline containing 0.1% Tween 20, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase for 1 h as described previously. Finally, the blots were washed and scanned on a densitograph.

**Statistical Analysis**

Results are presented as percentages of the control and represent the mean±standard error for 4 separate experiments performed in duplicate. Differences among all data were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by unpaired Student’s t-test. Differences of p<0.05 were considered statistically significant.

**Results**

**Effects of Homocysteine on MMP-2 in VSMCs**

Gelatin zymograms of VSMCs-conditioned media showed that the major MMP expressed under these conditions was MMP-2. Homocysteine (50–1,000\(\mu\)mol/L) increased the production of MMP-2 significantly in a dose-dependent manner and reduced the production of MMP-2 at high concentration (5,000\(\mu\)mol/L) (Fig 1A).

MMP-2 protein was expressed by Western blotting of the culture medium, probed using an anti-MMP-2 antibody. Homocysteine induced a significant and dose-dependent reduction of MMP-2 expression by Western blotting of culture medium probed using anti-MMP-2 antibody. Folic acid and magnesium decreased the MMP-2 protein significantly under these conditions in a dose-dependent manner (Figs 2A,3A). MMP-2 protein was expressed as shown by Western blotting of culture medium probed using anti-MMP-2 antibody. Folic acid and magnesium induced a significant and dose-dependent reduction of MMP-2 production by cells stimulated with homocysteine (Figs 2B,3B). The effect of folic acid on homocysteine-induced MMP-2 is better than that of magnesium and neither was toxic as determined by trypan blue exclusion.

**Changes in MMP-2 Production by VSMCs After Culture for 24, 48 or 72 h**

Production of MMP-2 under various treatments for 72 h increased more than during 24 or 48 h (Fig 4). Homocysteine significantly increased the production of MMP-2 in a time-dependent manner. Folic acid and magnesium significantly and dose-dependently decreased the production of homocysteine-induced MMP-2.

**Effects of Anions and Ions on Production of MMPs**

To verify that the altered homocysteine-induced MMP production by MgSO\(_4\) was caused by the magnesium ions and not a non-specific effect of sulfate anions, the effects of magnesium chloride on the production of homocysteine-induced MMP-2 were compared with those of MgSO\(_4\). No significant differences were seen in the degree of decrease in homocysteine-induced MMP-2 production between MgSO\(_4\) and magnesium chloride (data not shown), indicating that the effect on homocysteine-induced MMP-2 production was not caused by sulfate or chloride anions but...
by magnesium ions. To examine whether other divalent cations, such as zinc and copper, have any effects on homocysteine-induced MMP-2 production, VSMCs were treated with zinc sulfate or copper sulfate culture medium, which were made in the same way as the MgSO₄ treatments, to final concentrations of 0–500 μmol/L for 72 h. No effect on homocysteine-induced MMP-2 production by VSMCs treated with either zinc sulfate or manganese sulfate was seen (data not shown), indicating that the effect of magnesium on homocysteine-induced MMP-2 production in VSMCs was specific, not a common character of divalent cations.

Effects of Magnesium and Folic Acid on the Zymography System

To detect the effects of magnesium and folic acid on the zymography system, subconfluent VSMCs were treated with 0 mmol/L magnesium or folic acid for 24 h, then the 0 mmol/L culture medium was adjusted to different concentrations of magnesium (0–3.0 mmol/L) and folic acid (0–10 μmol/L) before zymography. Magnesium and folic acid did not influence MMP detection under these conditions. The lack of an inhibitory effect of magnesium and folic acid on homocysteine-induced MMP expression after removal of cells demonstrated the feasibility of this method for studying the effects of magnesium and folic acid.

Discussion

The extracellular MMPs, including gelatinase A (MMP-2), gelatinase B (MMP-9), metalloelastase (MMP-12), and matrilysin (MMP-7), are a family of distinct proteases with differing specificities for cleaving the various extracellular matrix components. It has been reported that the processes of migration and proliferation of VSMCs that contribute to the morphogenesis of atherosclerotic plaques require the remodeling of extracellular matrix by MMPs. The production of MMPs in VSMCs is regulated by a number of cytokines and growth factors, such as platelet-derived growth factor, secreted by platelets and vascular cells. Of the MMPs, MMP-2 has the widest distribution and plays an important role in the turnover of basement membrane type IV collagen and in controlling cell proliferation. The proliferation and migration of VSMCs is closely related to the stimulation of MMP-2 production and increased expression of MMP-2 has been revealed in atherosclerotic plaques. It has been shown that the binding of MMP-2 to insoluble elastin induces fast autoactivation of the proenzyme, suggesting that this mechanism could be relevant to the focal elastolysis that occurs in the arterial wall during arteriosclerosis. All these studies suggest that MMP-2 plays an important role in the formation and progression of atherosclerotic lesions.

Homocysteine is a non-protein-forming, sulfur-containing amino acid that stands at the crossroads of 2 metabolic pathways. Our previous study showed that homocysteine levels were elevated in patients with early CAD and with high risk factors. Hyperhomocysteinemia plays an important role in the pathogenesis of CAD. Indeed, the past decade has witnessed an exponential increase in studies defining plasma homocysteine as an independent risk factor, similar to smoking or hyperlipidemia, for atherosclerotic cardiovascular, cerebrovascular and peripheral vascular diseases. However, as yet little is known about the pathogenic mechanisms underlying the action of homocysteine itself.

We found in this study that low concentrations of homocysteine (from 50 μmol/L to 1,000 μmol/L) activated the production of MMP-2 and high levels of homocysteine (>5,000 μmol/L) inhibited it. Lee et al showed that high concentrations of homocysteine damaged both VSMCs and endothelial cells with respect to cell survival, proliferation and function. By increasing exposure to homocysteine, they showed that physiologic high concentrations of homo-
cysteine enhanced VSMCs proliferation. Bescond et al suggested that homocysteine exerted a dual effect, activating proMMP-2 at low molar ratio (MR 10:1) and inhibiting active MMP-2 at high MR (>1,000:1), and that the direct activation of proMMP-2 by homocysteine could be one of the mechanisms involved in the extracellular matrix deterioration in hyperhomocysteinemia-associated atherosclerosis.

The major finding of the present study is that extracellular magnesium and folic acid supplementation reduced the production of homocysteine-induced MMP-2 in rat VSMCs. To our knowledge, this is the first report of such effects. Clinically, Shibata et al reported that intravenous administration of magnesium decreased serum levels of MMP-1 in patients with acute myocardial infarction, which supports our finding. Long-term folic acid supplementation improves arterial endothelial function and has potential implications for the prevention of atherosclerosis in adults with hyperhomocysteinemia. Magnesium, a natural calcium antagonist, acts on potent-ial-organized placebo-control intervention study suggested that coronary endothelial function improves after treatment with folic acid and cobalamin. Folic acid supplementation in hyperhomocysteinemia is associated with decreases in both MR-1 and intraplatelet cGMP, and in the absence of an increase in the levels of the inflammatory mediator neopterin. Homocysteine exerts atherogenic effects in part by enhancing chemokine responses in cells involved in atherogenesis and folic acid supplementation may down-regulate these inflammatory responses.

Folate acts directly to produce antioxidant effects through its interactions with enzyme endothelial NO. Folate acts indirectly to lower homocysteine levels and ensure optimal functioning of the methylation cycle. Miller et al showed that folate acid treatment reduced, in part, the MMP activity at 66 and 72 kD (MMP-2) and concluded that even folic acid treatment alone may be sufficient for decreasing the negative effects of homocysteine. Hagar suggested that hyperhomocysteinemia aggravates myocardial infarction via oxidative stress mechanisms and that lowering the homocysteine level with folic acid and vitamin B12 can ameliorate the detrimental effects of hyperhomocysteinemia and may reduce the risk of myocardial infarction.

Magnesium, a natural calcium antagonist, acts on potential-operated channels, receptor-operated channels, and leak-operated channels in the cardiovascular system. We previously reported the effects of magnesium on the production of extracellular MMPs in cultured rat VSMCs and recently, magnesium has been shown to inhibit capacitative Ca2+-entry in VSMCs. Li et al suggested the need for the 3 B-vitamins (folic acid, vitamin B6 and B12), together with normal physiological levels of magnesium, in order to prevent [Mg2+]i depletion and occlusive cerebral diseases induced by hyperhomocysteinemia.

To date, there have not been controlled data on the effects of homocysteine-lowering treatment on vascular function or clinical endpoints. The precise mechanisms by which homocysteine mediates its adverse vascular effects are in fact unknown but may relate to impaired SMC function.

Conclusion

In cultured rat VSMCs, magnesium and folic acid significantly reduced the production of homocysteine-induced MMP-2 in a dose-dependent manner. Our data suggest that the beneficial effect of magnesium and folic acid supplementation on vascular disease processes may be related, at least in part, to their inhibitory effect on the production of homocysteine-induced MMP-2 in VSMCs.

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References


