

Effect of erythromycin on homocysteine-induced extracellular matrix metalloproteinase-2 production in cultured rat vascular smooth muscle cells

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Received May 11, 2004

Background & objectives: Several lines of evidence have shown an association between *Chlamydia* infection and atherosclerosis, but clinical trials of preventive antibiotic (erythromycin) treatment in patients with coronary artery disease have shown conflicting results. Hyperhomocysteinaemia is an independent risk factor of coronary artery disease and causes an intense remodelling of the extracellular matrix in arterial walls, particularly an elastolysis involving metalloproteinases. In the present study we investigated the effects of erythromycin on the production of homocysteine-induced extracellular matrix metalloproteinase-2 (MMP-2) in cultured rat vascular smooth muscle cells (VSMCs).

Methods: Effects of different concentration of homocysteine (Hcy) (0-5000 $\mu\text{mol/l}$) on MMP-2 production, and the effects of different erythromycin concentrations (0-10 mmol/l) on homocysteine-induced MMP-2 production in cultured rat VSMCs were studied using gelatin zymography and Western blotting. The changes of MMP-2 under various treatments for 1, 3 and 5 days were also compared.

Results: Homocysteine (50-1000 $\mu\text{mol/l}$) increased the production of MMP-2 significantly in a dose-dependent manner and reduced the production of MMP-2 at a high level (5000 $\mu\text{mol/l}$). Increased production of MMP-2 induced by homocysteine was reduced by extracellularly added erythromycin in a dose-dependent manner.

Interpretation & conclusions: Homocysteine increased the production of MMP-2 significantly in a dose-dependent manner. Extracellularly added erythromycin decreased homocysteine-induced MMP-2 secretion. The findings of the present study suggested that the beneficial effect of erythromycin on vascular disease processes might be due to its inhibitory effect on the Hcy-induced production of MMP-2 in VSMCs.

Key words Atherosclerosis - erythromycin - homocysteine matrix metalloproteinase - smooth muscle cell

Coronary artery disease (CAD) is one of the most common fatal diseases in the developed world, and its incidence is increasing in developing countries. Though various factors may initiate the atherosclerotic process, inflammation with activation of macrophages and T lymphocytes, is central¹. Several lines of evidence have led to the association between infection with *Chlamydia* and atherosclerosis. In 1988, a serological association was reported between coronary heart disease and antibodies to *Chlamydia pneumoniae*². Numerous reports since then, have confirmed this association. In addition, multiple studies of atherosclerotic plaques have found evidence of *C. pneumoniae* by immunohistochemical stains, polymerase chain reaction analysis, or culture³. Animal model studies have confirmed the development of atherosclerosis after respiratory tract inoculation with *Chlamydia*⁴. Pilot clinical trials of preventive antibiotic (erythromycin) treatment in patients with coronary artery disease have shown conflicting results^{5,6}.

Hyperhomocysteinaemia is known to be an independent risk factor of coronary artery disease. Homocysteine (Hcy) contributes, in part, to enhanced vascular inflammation and hypercoagulability, factors intimately linked to the development of atherosclerosis and associated thrombotic events⁷⁻⁹. Hyperhomocysteinaemia causes an intense remodelling of the extracellular matrix in arterial walls, particularly an elastolysis involving metalloproteinases¹⁰. Four metalloproteinases belonging to the matrix metalloproteinases (MMP) family are known as elastolytic proteinases: gelatinase A (MMP-2), gelatinase B (MMP-9), metalloelastase (MMP-12), and matrilysin (MMP-7). Only MMP-2 and MMP-9 are expressed as latent proenzymes by aortic smooth muscle cells and both are involved in arterial diseases such as atherosclerosis¹¹. 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 in the arterial wall during arteriosclerosis¹⁰.

However, the effect of erythromycin on Hcy-induced MMP-2 production in cultured vascular smooth muscle cells (VSMCs) has not been studied. The present study was undertaken to test whether

extracellularly added erythromycin alters MMP-2 secretion at the basal and in Hcy stimulated conditions in rat VSMCs.

Material & Methods

Chemicals: 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, New York), foetal calf serum (FCS) from Filtron Pty Ltd (Tokyo, Japan), D, L-homocysteine from Nacalai Tesqu Inc. (Kyoto, Japan), rabbit anti-MMP-2 antibody (NeoMarkers antibody line, Ab-7, RB-1537-PO) from Lab Vision Co. (Westinghouse Drive - Fremont, CA, USA). Erythromycin was purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). All other chemicals were of reagent grade.

Preparation of smooth muscle cells: Rat aortic smooth muscle cells were isolated by enzymatic digestion from the thoracic aortic of 6-wk-old male Sprague Dawley rats (n=5) (Charles River Japan, Kanagawa, Japan) as described previously¹². All surgical interventions and anesthesia were conducted in conformity with institutioned guidelines. The animal experimentation was approved by the ethics committee of Fukui Medical University. The cells were cultured in DMEM supplemented with 10 per cent foetal calf serum at 37°C in a humidified 5 per cent CO₂: 95 per cent 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 (Muscle Specific Actin, Enzo, Farmingdale, New York, USA), a monoclonal antibody that recognizes muscle-specific actin. All VSMCs 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 cells were exposed to various treatments.

Preparation of culture medium: Hcy was added to DMEM without magnesium to the final Hcy concentrations of 0-5000 µmol/l (µM); the final concentrations of erythromycin were 0-10 mmol/l (mM) before use. The level of 6.25-12.5 µmol/l Hcy is physiological and > 25 µmol/l is pathological. All treatments of erythromycin were sustained in the 100 µM Hcy-treated media for 5 days.

Analysis of gelatinase production: After various treatments (treatment with homocysteine, homocysteine + erythromycin in different concentration) for 1, 3 and 5 days, medium samples were harvested, centrifuged at $2000\times g$ for 10 min and normalized for cell protein content using Bio-Rad assay¹². The samples were applied without reduction to a 7.5 per cent 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 $\mu\text{mol/l}$ ZnCl_2 , and 2.5% Triton X-100), then incubated overnight at 37°C with shaking in the same buffer containing 1 per cent rather than 2.5 per cent Triton X-100. The gel was stained with a solution of 0.1 per cent 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 analysis: After various treatments for 1, 3 and 5 days, medium samples were harvested with the protease inhibitors phenylmethane sulphonyl fluoride (0.1 mmol/l) and leupeptin (10 $\mu\text{g/ml}$) from cells, centrifuged at $2000\times g$ for 10 min and separated by electrophoresis on 7.5 per cent sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) followed by transfer onto polyvinylidene difluoride membranes (Immobilon P, Millipore, Japan 0.22 μm pore size). The membranes were blocked in 5 per cent skim milk in phosphate-buffered saline containing 0.1 per cent Tween-20 (PBST) at room temperature for 1 h, and probed with anti-MMP-2 monoclonal antibody overnight. After washing three times with PBST, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase for 1 h as described previously¹³. Finally, the blots were washed and scanned on a densitograph.

Statistical analysis: Results were presented as percentages of the control and represent the mean \pm standard errors (SE) for four separate experiments performed in duplicate. The duplicate studies gave consistent results. 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.

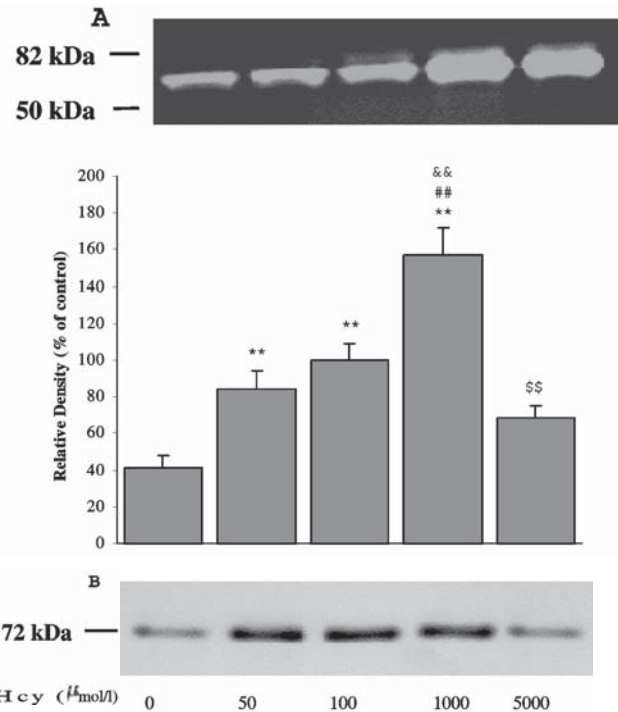


Fig. 1. Effects of Hcy on production of MMP-2 in cultured vascular smooth muscle cells. Hcy (50-1000 $\mu\text{mol/l}$) significantly increased the production of MMP-2 as determined by zymography (A) and Western blotting (B) in a dose-dependent manner. Clear zones against the blue background indicated the presence of MMP-2. Columns indicate data of gelatinolytic activity as percentages of the amount of control (100 $\mu\text{mol/l}$ Hcy), and represent the mean \pm SE for five separate experiments performed in duplicate. ** $P < 0.01$ vs Hcy 0 $\mu\text{mol/l}$; # $P < 0.05$ vs Hcy 50 $\mu\text{mol/l}$, ## $P < 0.01$ vs Hcy 50 $\mu\text{mol/l}$; \$ $P < 0.05$ vs Hcy 100 $\mu\text{mol/l}$; \$\$ $P < 0.01$ vs Hcy 100 $\mu\text{mol/l}$.

Results

Effects of homocysteine on MMP-2 in VSMCs: Gelatin zymograms of VSMCs-conditioned media showed that major MMP expressed under these conditions was MMP-2. Hcy (50-1000 $\mu\text{mol/l}$) increased the production of MMP-2 significantly in a dose-dependent manner (Fig. 1A), and reduced the production of MMP-2 at high Hcy concentration (5000 $\mu\text{mol/l}$).

MMP-2 protein was shown to be expressed by Western blotting of culture medium, probed using an anti-MMP-2 antibody. Hcy induced a significant and dose-dependent upgrade in MMP-2 production (Fig. 1B).

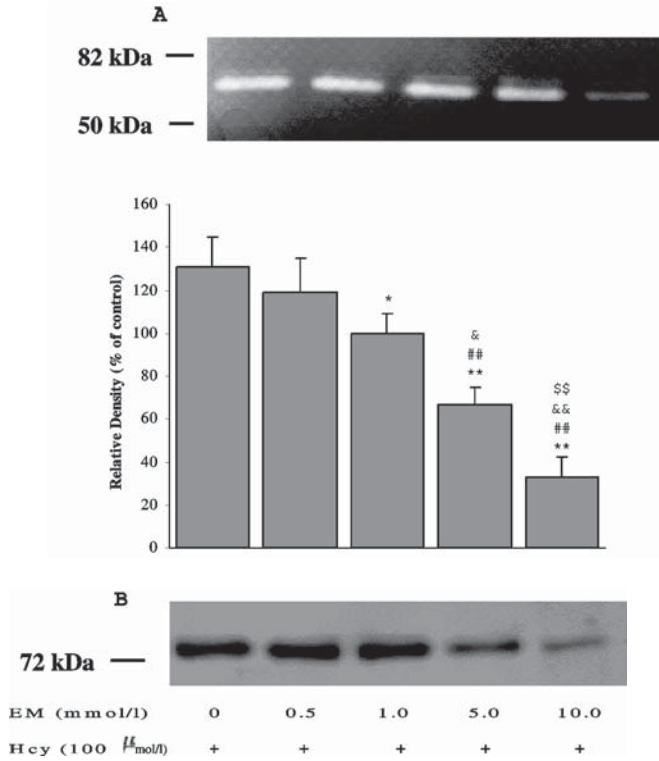


Fig. 2. Effects of erythromycin (EM) on production of MMP-2 stimulated by Hcy (100 μmol/l) in cultured vascular smooth muscle cells. Erythromycin significantly decreased the production of MMP-2 in a dose-dependent manner as determined by zymography (A) and Western blotting (B) under conditions of stimulation by Hcy. Clear zones against the blue background indicate the presence of MMP-2. Columns are as described in Fig. 1. Treatment with 1.0 mmol/l EM together with Hcy was regarded as control. * $P < 0.05$; ** $P < 0.01$ vs EM 0 mmol/l; ## $P < 0.01$ vs EM 0.5 mmol/l; & $P < 0.05$; && $P < 0.01$ vs EM 1.0 mmol/l; \$\$ $P < 0.01$ vs EM 5.0 mmol/l.

Effects of erythromycin on the production of homocysteine-induced MMP-2 in VSMCs: Effects of erythromycin (EM) on cells stimulated with Hcy (100 μmol/l) were examined. Erythromycin decreased the MMP-2 production significantly under these conditions in a dose-dependent manner (Fig. 2A). MMP-2 protein was shown to be expressed by Western blotting of culture medium probed using anti-MMP-2 antibody. Erythromycin induced a significant and dose-dependent reduction of MMP-2 production by cells stimulated with Hcy (100 μmol/l) (Fig. 2B). These effects were not toxic as determined by trypan blue exclusion.

Changes of MMP-2 after VSMCs cultured for 1, 3 and 5 days: Production of MMP-2 under various

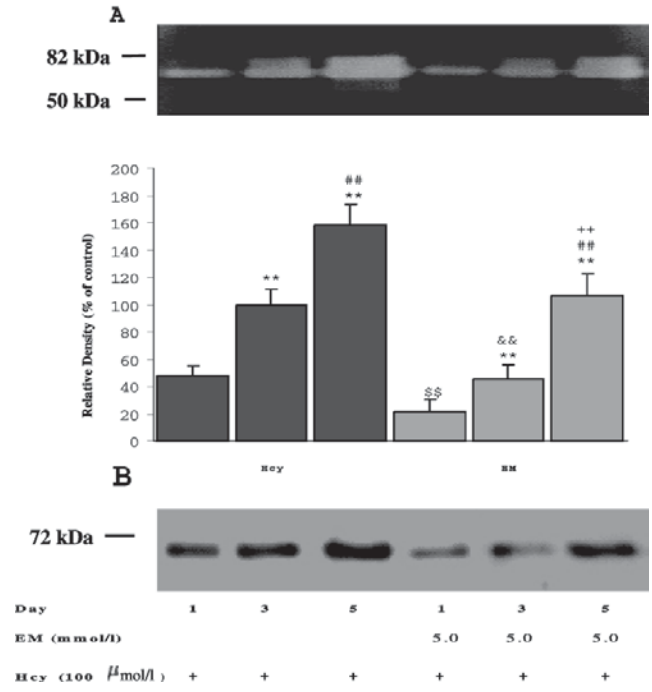


Fig. 3. Changes of MMP-2 levels after VSMCs cultured under various treatments for 1, 3 and 5 days. Hcy significantly increased the production of MMP-2 in a time-dependent manner. Erythromycin (EM) (5 mmol/l) significantly decreased the production of Hcy-induced MMP-2 in a dose-dependent manner as determined by zymography (A) and Western blotting (B). Clear zones against the blue background indicate the presence of MMP-2. Columns are as described in Fig. 1. Treatment with 500 μmol/l Hcy (3rd day) was regarded as control. ** $P < 0.01$ vs 1 day; ## $P < 0.01$ vs 3 days; compared with Hcy; \$\$\$ $P < 0.01$ vs 1 day; && $P < 0.01$ vs 3 days; ++ $P < 0.01$ vs 5 days.

treatments for 5 days increased more than 1 and 3 days (Fig. 3). Hcy significantly increased the production of MMP-2 in a time-dependent manner. Erythromycin significantly and decreased the production of Hcy-induced MMP-2 in a dose-dependent manner.

Effects of erythromycin on the zymography system: To detect the effects of erythromycin on the zymography system, subconfluent VSMCs were treated with 0 mmol/l erythromycin for 72 h, then the 0 mmol/l culture medium was adjusted to different erythromycin concentrations (0-10 mmol/l) before zymography. Erythromycin did not influence MMP detection under these conditions. The lack of an inhibitory effect of erythromycin on MMP expression after removal of cells demonstrated the feasibility of studying the effects of erythromycin.

Discussion

The extracellular MMPs are a family of distinct proteases with differing specificities of cleaving toward various extracellular matrix components¹¹. It has been reported that the processes of migration and proliferation of VSMCs that contribute to the morphogenesis of atherosclerotic plaques require the extracellular matrix remodeling caused by MMPs¹⁴. The production of MMPs in VSMCs is known to be regulated by a number of cytokines and growth factors such as platelet-derived growth factor (PDGF) secreted by platelets and vascular cells¹⁵. Among 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 proliferation¹¹. The proliferation and migration of VSMCs was demonstrated to be closely related to the stimulation of MMP-2 production¹². An increased expression of MMP-2 has been revealed in atherosclerotic plaques¹³. 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 in the arterial wall during arteriosclerosis. All these studies suggest that MMP-2 plays an important role in the formation and progression of atherosclerotic lesion.

While *in vitro* studies have revealed a number of Hcy-mediated alterations in the thromboregulatory properties of endothelial cells, comparatively little is known about Hcy-modulated smooth muscle cell function. Woo *et al*¹⁶ suggested that D,L-Hcy stimulation of bovine aortic smooth muscle cells proliferation involved mitogen-activated protein (MAP) kinase activation. An initial effect of Hcy is to induce release of intracellular Ca²⁺ in VSMCs and may induce vascular reactivity. The transient change in Ca²⁺ correlated with the effect on extracellular matrix associated with Hcy¹⁷. Desai *et al*¹⁸ suggested that Hcy might increase monocyte recruitment into developing atherosclerotic lesions by upregulating MCP-1 and interleukin-8 (IL-8) expression in VSMCs. Hyperhomocysteinaemia is associated with a significant decrease of the smooth muscle cell/extracellular matrix ratio of the media of muscular femoral arteries without significant changes in medial

thickness¹⁹. Taha *et al*²⁰ suggested that Hcy induces smooth muscle cell growth by a hydrogen peroxide-independent pathway and that the effects of Hcy may sum together with the known initiating events produced by oxidative stress and accelerate the progression of atherosclerosis. Tsai JC *et al*²¹ showed that Hcy and serum increased DNA synthesis synergistically in both human and rat aortic smooth muscle cells.

We found in this study that low concentration of Hcy (from 50 µmol/l to 1000 µmol/l) activated the production of MMP-2 and high levels of Hcy (5000 µmol/l) inhibited MMP-2. High concentrations of Hcy are shown to damage both VSMCs and endothelial cells with respect to cell survival, proliferation and function²². By increasing concentration of Hcy, it was shown that physiological high concentrations of Hcy enhanced VSMCs proliferation²². Hcy was shown to exert a dual effect, activating proMMP-2 at low molar ratio (MR 10:1) and inhibiting active MMP-2 at high molar ratio (MR>1000:1), and the direct activation of proMMP-2 by Hcy could be one of the mechanisms involved in the extracellular matrix deterioration in hyperhomocysteinaemia-associated arteriosclerosis¹⁰.

The major finding of this study was that extracellular erythromycin supplementation reduced the production of Hcy-induced MMP-2 in rat VSMCs. Data from basic laboratory studies including cells and animal models, and from patients have strongly suggested a possible role of *C. pneumoniae* in the aetiology of atherosclerosis. On the basis of these findings, antimicrobial treatment studies were done, with the intention to improve the clinical condition of patients with coronary artery disease (CAD) by chlamydial eradication from atheromatous plaques²³. Endothelial and smooth muscle cells can acquire a productive chlamydial infection that can be inhibited *in vitro* by common antichlamydial antibiotics. Parchure *et al*²⁴ showed a favourable effect of azithromycin treatment on endothelial function in patients with coronary artery disease and evidence of *C. pneumoniae* infection irrespective of antibody titre levels. Muhlestein *et al*²⁵ were the first to demonstrate a protective effect of antibiotic therapy against accelerated atherosclerosis in a rabbit model. Gurfinkel *et al*²⁶ reported a 7 per cent absolute reduction in the combined end point of cardiac

ischaemic death, myocardial infarction, and severe recurrent angina after 30 days of treatment with roxithromycin (ROXIS trial). An absolute risk reduction of 14.9 per cent was shown in the secondary end point (combination of death, myocardial infarction, unstable angina, ischaemic stroke, and critical limb ischaemia) although no significant effect of antibiotic therapy was noted in the primary end point (death, myocardial infarction or unstable angina within the 3-month treatment period)²⁷. In the St. George's Hospital trial, patients receiving azithromycin had a similar outcome as those with negative chlamydial serology²⁸. Stone *et al*²⁹ showed that patients receiving antibiotic therapy had a 36 per cent reduction in the incidence of cardiac death, unstable angina, or myocardial infarction noted at 12 wk, and the reduction persisted throughout the 1-yr follow-up (STAMINA trial). More evidence in favour of a role of *C. pneumoniae* emerged from basic laboratory studies, whereas clinical trials of patients with stable and unstable coronary artery disease failed to demonstrate a significant benefit of antibiotic therapy.

Up till now, there have been no controlled data on the effects of Hcy treatment on vascular function or clinical end points. The precise mechanisms by which Hcy mediates its adverse vascular effects are in fact unknown, but may relate to impaired smooth muscle cell function.

In conclusion, in cultured rat VSMCs, erythromycin significantly reduced the production of Hcy-induced MMP-2 in a dose-dependent manner. Our findings suggested that the beneficial effect of erythromycin supplementation on vascular disease processes may be due, at least in part, to the inhibitory effect of erythromycin on the production of Hcy-induced MMP-2 in VSMCs.

Acknowledgment

This study was supported in part by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Science and Culture of Japan. Authors thank Drs Hiromasa Shimizu, Hiroshi Tsutani, Yasuhiko Mitsuke and Hiromichi Iwasaki for data collection and assistance in statistical analysis, and Miss Hiromi Nishimura and Makoto Matsumoto for technical assistance.

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