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Original Article

Folic acid reduces chemokine MCP-1 release and expression in rats with hyperhomocystinemia

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Abstract

Objective: This study aimed to investigate the effects of folate on the monocyte chemoattractant protein-1 (MCP-1) expression and release in rats with hyperhomocystinemia induced by ingestion of excess methionine. **Methods and results:** Thirty male Sprague–Dawley rats (200±20 g) were randomly divided into three groups (n=10 for each group): control group (Control), high-homocystinemia (Hhcy) group, and folate treatment (FA) group. They were fed with a normal regular diet, enriched by 1.7% methionine plus 1.7% methionine and 0.006% folate for 45 days. Our study showed the following: (a) A high methionine diet for 45 days is sufficient to induce hyperhomocystinemia; folate supplementation to the rats fed the high-methionine diet prevented an elevation homocysteine (Hcy) levels in the blood (P<.01). (b) Compared with the Control group, the Hhcy group had elevated plasma levels of MCP-1, and Hcy was significantly correlated with MCP-1 (P<.05). (c) The protein and mRNA expression of MCP-1 in the aorta was higher in rats from the Hhcy group than in rats from the Control group. (d) Most important, after folic acid supplementation, the lowering of Hcy levels was accompanied by a marked reduction of MCP-1 expressed in aortae and released from plasma and peripheral blood mononuclear cells (PBMCs) stimulated by oxidized low-density lipoprotein (P<.05, P<.01). **Conclusion:** Folic acid supplementation not only can blunt the rise in Hcy and reduce MCP-1 released from both plasma and PBMCs of rats with hyperhomocystinemia but also can downgrade MCP-1 expression in the aorta of rats with hyperhomocystinemia.

Keywords: Folic acid; MCP-1; Hyperhomocystinemia

1. Introduction

Homocystinemia is an independent risk factor for atherosclerosis. Several plausible mechanisms for Hcy-induced atherosclerosis have been proposed. These include endothelial dysfunction, enhancement of oxidative stress, reduction in NO bioavailability, and augmentation of thrombus formation [1–4]. However, the precise molecular mechanism is still unclear.

There is considerable evidence that atherogenesis is an inflammatory process [5]. Cytokines play critical roles as multipotent mediators of inflammation by modulating key functions of vascular cells [5,6]. Dysfunction of endothelial cells is the key process promoting inflammatory reactions. On injury, endothelial cells are capable of producing various cytokines that participate in inflammatory reactions in the arterial wall. Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that stimulates migration of monocytes into the intima of arterial walls [7–11]. The amount of this chemokine appears to be increased in atherosclerotic lesions in both human and experimental animals [12,13].

Although results from in vitro studies suggest that Hcy, at pathophysiological concentrations, stimulates chemokine expression in vascular cells [14–16], it is unknown whether hyperhomocystinemia can initiate similar changes, leading to enhanced monocyte adhesion/binding to the vascular endothelium in vivo. On the basis of the potential pathogenic role of chemokines in atherogenesis, the objective of the present study was to investigate whether

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homocysteine (Hcy) may exert its effect, in part, through MCP-1 and whether folic acid supplementation may down-regulate these inflammatory responses.

2. Materials and methods

2.1. Materials

2.1.1. Animal model

Male Sprague–Dawley rats (bred from animal centers of Tongji Medical College, Huazhong Science and Technology University), aged 8 weeks, were divided into three groups (n=10 for each group) and maintained for 45 days on the following diets before the experiments: (a) the Control group was fed with regular diet; (b) the high-homocystinemia (Hhcy) group was fed with high-methionine diet, consisting of regular diet plus 1.7% methionine; and (c) the folate treatment (FA) group was fed with high-methionine plus folate-rich diet, consisting of regular diet plus 1.7% methionine and 0.006% folate [17]. Plasma and serum samples were collected and stored at −80°C after 45 days until analysis.

2.2. Methods

2.2.1. Plasma Hcy measurement

The plasma Hcy concentration was determined via high-pressure liquid chromatography [18].

2.2.2. Isolation and oxidation of LDL

Native human low-density lipoprotein (LDL) (density=1.03–1.063 g/ml) was isolated from fresh plasma of normal lipidemic donors by sequential ultracentrifugation and was oxidized in the presence of freshly prepared 5 μmol/l $CuSO_4$ (final concentration) as described previously [18,19]. Oxidized LDL (oxLDL), used in our experiments, contained 347 nmol of lipid peroxides per milligram of LDL protein, and relative electrophoretic mobility was 4.0. oxLDL was stored under N_2 and used within 2 weeks.

2.2.3. Release of chemokine from PBMCs

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood of rats by gradient centrifugation in Isopaque-Ficoll (Lymphoprep, Jingmei Biotech Co., Ltd.) within 45 min and were incubated in flat-bottomed 24-well trays (1×10^6/ml, 200 μl per well) in medium alone [RPMI1640 medium (Sigma Chemical Co.) containing 2 mmol/l L-glutamine, 100 U/ml penicillin, and 5% autologous serum] or with oxLDL (final concentration, 100 μg/ml). Cell-free supernatants were harvested after culture for 48 h, divided into aliquots, and stored at −80°C until analysis.

2.2.4. Enzyme immunoassays

The concentration of MCP-1 in plasma and in cell-free supernatants was measured via ELISA according to the manufacturer's protocols (R&D Systems, Minneapolis, MN, USA).

2.2.5. Immunohistochemistry for the aorta of rats

The thoracic aorta was isolated and divided into segments to detect the endothelial expression of MCP-1. These segments were immersion fixed in 10% neutral-buffered formalin overnight and then embedded in paraffin. Sequential 5-μm paraffin-embedded cross sections were prepared. Immunohistochemical analysis was performed to detect MCP-1. The fixed cryosections were immediately blocked in 10% horse serum and phosphate-buffered saline (PBS) at room temperature for 30 min. Rabbit polyclonal antibodies against rat MCP-1 (Santa Cruz Biotechnology) were diluted 1:100 in PBS and incubated with the cryosections for 1 h at room temperature. After three washes, the sections were incubated with biotin-conjugated antirabbit immunoglobulins (Dako) at 1:250 dilution in PBS. After three washes, the samples were mounted in 90% glycerol–PBS. Photographs were taken by use of a light microscope at a magnification of ×200.

2.2.6. Western immunoblotting for detection of MCP-1 protein in the aorta

To investigate the effects of supplementation of folic acid on the expression of MCP-1 in aortae of rats with hyperhomocystinemia, we performed Western immunoblotting analyses. In brief, the total cellular protein in aortae of rats in three groups were extracted by 50 mmol/l Tris–HCl (pH 7.4). Samples containing 30 μg of total protein were separated on an SDS-11% polyacrylamide gel followed by electrophoretic transfer of separated proteins to nitrocellulose membrane. The nitrocellulose was probed with primary rabbit antibodies that recognized rat MCP-1 (1:500, Santa Cruz Biotechnology). The blots were developed using horseradish-peroxidase-conjugated secondary antibodies (1:5000, New England BioLabs). Bands of interest were visualized using enzyme chemiluminescence reagents (Amersham). The band intensities were quantified via densitometry. β-Actin served as internal standard.

2.2.7. Semiquantitative RT-PCR for detection of MCP-1 mRNA in the aorta of rats

Total RNA was isolated via guanidinium thiocyanate–phenol–chloroform extraction. The mRNA was reverse transcribed from 1 to 5 μg total RNA using 10 μmol/l Oligo (dT)12–18 1-μl primers and Superscript (Invitrogen). MCP-1 gene expression was then estimated via semiquantitative PCR using a sense primer (5′-CAGGTCTCTGTCA-CGCTTCT-3′) and an antisense primer (5′-AGTATTCATG-GAAGGGAATAG-3′) to yield MCP-1 (523 bp). Typically, the amplification was carried out for 30 cycles with an annealing temperature of 72°C. The concentration of the reverse-transcribed cDNA in the PCR mixture was adjusted to assure a linear correlation between template and product. The amplified DNA was analyzed via agarose gel