Homocysteine induces production of monocyte chemoattractant protein-1 and interleukin-8 in cultured human whole blood

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ABSTRACT

AIM: To investigate whether increased plasma L-homocysteine (Hcy) level could promote monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) in cultured whole blood. METHODS: Human whole blood or different type of peripheral blood cells from health volunteers were incubated with Hcy and/or the inhibitors. MCP-1 and IL-8 level were measured by ELISA assay. RESULTS: Hcy 10-1000 µmol/L induced production of MCP-1 and IL-8 in cultured human whole blood (P<0.05). The major cellular source of these chemokines comed from monocytes. Meanwhile,Hcy also promoted the upregulation of MPO level even at the 10 µmol/L in the cultured whole blood. The intracellular ROS, particular the OH• radicals, play extremely important role in the Hcy-induced MCP-1 and IL-8 production. CONCLUSION: Increased Hcy level in plasma (hyperhomocysteinemia) induced MCP-1 and IL-8 secretion in cultured human whole blood, especially in monocytes via oxidative stress mechanism.

INTRODUCTION

Homocysteine is an amino acid formed during the metabolism of methionine. Hyperhomocysteinemia, the elevated plasma Hcy levels, was defined as an independent risk factor for coronary heart disease and thrombosis[1,2]. Whether hyperhomocysteinemia is causal or merely a marker for cardiovascular disease is subject of investigation.

Chemokines belong to a superfamily of structure-related small chemotactic cytokines involved in leukocyte trafficking. Monocyte chemoattractant protein-1 (MCP-1), a prototype of CC chemokine[3], and interleukin-8 (IL-8), a prototype of CXC chemokine[4], were crucial in converting rolling monocytes to firm adhesion on endothelial monolayers and played pivotal role in the systemic thrombosis formation[5-9]. This strongly implied that increased plasma MCP-1 and IL-8 might promote the progression of atherosclerotic and thrombotic lesions.

Hcy could promote the expression and secretion of MCP-1 and IL-8 from cultured human aortic endothelial cells[10,11], MCP-1 from a human smooth muscle cell line[12]. All the studies suggested that Hcy could...
promote the local plaque development by increasing the MCP-1 and IL-8 level locally.

The current study was to investigate whether increased plasma Hcy level could promote the MCP-1 and IL-8 level in cultured whole blood. If true, it would indicate directly that hyper-homocysteinemia was not only the mark but also the vital causation of vascular disease development.

MATERIALS AND METHODS

Human whole blood culture The investigation conformed to the principles outlined in the Declaration of Helsinki. The human whole blood cultures were slightly modified from our previous studies. Briefly, blood from healthy donors was drawn into heparinized syringes. Part of whole blood was then placed on a rotator and incubated in an atmosphere containing 5% CO₂ at 37 °C. The other portion of the whole blood was separated into primary cultures of monocytes, lymphocytes, and neutrophils. To prepare these, we used the density gradient from Nycoprep 1.077 (Life Technologies, Grand Island, NY). Blood was layered over the gradient, and the cells were separated by centrifugation. Peripheral blood mononuclear cells (PBMC) and neutrophils were obtained respectively. Monocytes were then enriched from PBMC by adherence to serum-coated culture flasks for 2 h. Adherent cells (monocytes) and suspended cells (lymphocytes) were collected. Isolated cells were plated in flat bottom of 96-well culture plates at a density of 5×10⁵ (monocytes), 1.5×10⁶ (lymphocytes), and 5×10⁶ (neutrophils). Cell viability was evaluated by Trypan blue exclusion. Only cell preparations with a 95% or greater viability were used.

Measurement of MCP-1 and IL-8 protein secretion Human whole blood was treated with Hcy for indicated times and/or preincubated for 60 min with antioxidants including De₃SO, catalase (CAT) 250 kU/L, catalase-polyethylene glycol (PEG-CAT) 250 kU/L, superoxide dismutase (SOD) 500 kU/L or superoxide dismutase-polyethylene glycol (PEG-SOD) 500 kU/L or other pharmacological reagents. The plasma was harvested and transferred to other polypropylene tubes and stored at -30 °C for not more than 1 week before measurement of chemokines and cytokines. TNF-α, IL-1β, MCP-1, and IL-8 protein concentrations in the plasma were determined by ELISA (R&D Systems Inc, Minneapolis, MN).

Measurement of LDH release Cultured human whole blood was treated with Hcy 10-1000 µmol/L for 32 h. Cell suspensions were collected for determination of LDH. LDH activity was measured by spectrophotometric enzyme activity method and expressed as units per liter medium.

Chemicals L-Hcy, L-cysteine, and L-methionine, were purchased from Sigma Co (St Louis, MO). RPMI-1640 was purchased from Gibco Laboratories (Grand Island, NY). Other chemicals were purchased from the Chinese Chemical Co (Beijing, China).

Statistical methods Results were expressed as mean±SD. The number of samples used for each group was presented in the figure legends. The data were analyzed using one-way ANOVA and further analyzed using the Student-Newmen-Keuls test for multiple comparisons within treatment groups or t-test (unpaired test with Welch’s correction) for comparison between two groups with non-normal distribution. P<0.05 was considered significant between treatment groups.

RESULTS

Effect of Hcy on MCP-1 and IL-8 levels from cultured human whole blood Hcy induced the secretion of MCP-1 and IL-8 above basal levels within 4 h, and the chemokine levels continued to increase throughout the remainder of the time course (Fig 1A and 1B). When human whole blood was treated with increasing concentrations of Hcy (10-1000 µmol/L) for 32 h, Hcy as little as 10 µmol/L increased the secretion of MCP-1 production which was higher than that at Hcy 100 µmol/L (Fig 2A). However, the difference between Hcy 1000 µmol/L group and control group was not significant. The concentration-dependent effect of Hcy on IL-8 protein production was slightly different (Fig 2B) since it reached the maximal level at Hcy 100 µmol/L (Fig 2A). However, the difference between Hcy 1000 µmol/L group and control group was not significant. The concentration-dependent effect of Hcy on IL-8 protein production was slightly different (Fig 2B) since it reached the maximal level at Hcy 100 µmol/L and there was no reduction at Hcy 1000 µmol/L.

Effect of Hcy on secretion of MPO in cultured human whole blood Hcy 100 µmol/L induced enhancement of MPO secretion above basal levels since 16 h later (Fig 3A). When human whole blood was treated with increasing concentrations of Hcy (10-1000 µmol/L) for 32 h, Hcy as little as 10 µmol/L increased the secretion of MPO-1 production which was higher than that at Hcy 100 µmol/L (Fig 2A). However, the difference between Hcy 1000 µmol/L group and control group was not significant. The concentration-dependent effect of Hcy on IL-8 protein production was slightly different (Fig 2B) since it reached the maximal level at Hcy 100 µmol/L and there was no reduction at Hcy 1000 µmol/L.

Effect of Hcy on LDH release from cultured human whole blood To test whether the concentrations of Hcy used in the experiment have any toxic effect to the cultured whole blood cells, the LDH concentration of plasma from the whole blood incubated
Fig 1. Time course of Hcy 100 µmol/L on secretion of MCP-1 (A) and IL-8 (B) in cultured human whole blood. n=5 independent experiments. Mean±SD. bP<0.05 vs corresponding untreated groups.

Fig 2. Effect of Hcy on secretion of MCP-1 (A) and IL-8 (B) in cultured human whole blood. n=5 independent experiments. Mean±SD. bP<0.05 vs corresponding untreated groups.

Fig 3. Effect of Hcy on the secretion of MPO in cultured human whole blood. (A) Whole blood was incubated with Hcy 100 µmol/L for different periods; (B) Whole blood was treated with different concentrations of Hcy (10-1000 µmol/L) for 32 h. n=5 independent experiments. Mean±SD. bP<0.05 vs corresponding untreated groups.

Fig 4. Effect of Hcy on LDH release from cultured human whole blood. Whole blood was incubated with various concentrations of Hcy for 32 h. n=5 independent experiments. Mean±SD. bP<0.05 vs corresponding untreated groups.

Compared with the control, the Hcy concentrations used in our study have no significant toxic effect to cultured blood cells (Fig 4).
Major cellular source of Hcy-induced secretion of MCP-1 and IL-8 from human whole blood. After the isolated monocytes, lymphocytes, and neutrophils were treated with Hcy 100 µmol/L for 32 h, respectively, MCP-1 and IL-8 secretion were increased only in cultured monocytes. Hcy did not induce MCP-1 and IL-8 secretion from lymphocytes or neutrophils. These data suggested that the monocytes were principally responsible for the Hcy-induced MCP-1 and IL-8 secretion in cultured human whole blood (Fig 5).

Role of ROS in Hcy-induced secretion of MCP-1 and IL-8 from human whole blood and monocytes. To test the role of ROS in Hcy-induced chemokine secretion, whole blood and monocytes were pretreated for 60 min with antioxidant reagent 1/1000 De2SO and 1/100 De2SO (a scavenger of OH•), respectively. The cultured whole blood was then stimulated with Hcy 100 µmol/L for 32 h. De2SO 1/100 significantly inhibited Hcy-induced secretion of both MCP-1 and IL-8 in whole blood (Fig 6A and 6B). In addition, whole blood was pretreated with antioxidant such as scavengers of H2O2, CAT and polyethylene glycol-CAT (PEG-CAT) and scavengers of O2•−, SOD and polyethylene glycol-SOD (PEG-SOD) for 60 min, respectively. The cultured whole blood was then stimulated with Hcy 100 µmol/L for 32 h. PEG-SOD and PEG-CAT significantly inhibited Hcy-induced secretion of MCP-1 and IL-8 in human whole blood (Fig 7A and 7B). However, SOD and CAT had no such effects. Compared with SOD and CAT, polyethylene glycol which was conjugated with SOD and CAT increased enzyme ability into intracellular part in cultured cell[15]. The data suggested that Hcy-induced ROS were produced intracellularly.

DISCUSSION

The major findings of this study were that increased Hcy level in the cultured whole blood led enhancement of MCP-1 and IL-8 level. The monocytes were principally responsible for the Hcy-induced MCP-1 and IL-8 secretion in cultured human whole blood. The intracellular ROS played critical role in the Hcy-induced MCP-1 and IL-8 production. Moreover,
elevated Hcy level in plasma also promoted the MPO release in the cultured whole blood.

Increased plasma MCP-1 and IL-8 levels played the pivotal role in the progression of vascular diseases and thrombosis\[6-9,16,17\]. Our findings provided the direct evidence to answer the question whether hyperhomocysteinemia was only cardiovascular diseases marker or the vital mediator.

Previous investigations showed that the mild hyperhomocysteinemia (10-300 µmol/L) was obviously related with increased vascular disorders\[18-25\]. To clarify whether the Hcy-mediated MCP-1 and IL-8 production was enhanced significantly and reached the maximum level even Hcy concentration was as low as 10 µmol/L. The data suggested that the Hcy-mediated MCP-1 synthesis was much more sensitive than IL-8. Considering the Hcy levels in the most of hyperhomocysteinemia patients’ plasma was in the mild range (10-30 µmol/L), our data suggested that MCP-1 might be responsible for more important pathogenesis role than IL-8 in the most of hyperhomocysteinemia patients.

The major blood cells include the monocytes, lymphocytes, and neutrophils. To find the cellular resource of MCP-1 and IL-8 synthesis in whole blood under Hcy action, such blood cells were isolated and treated with Hcy respectively. Our data demonstrated that only the monocyte-derived MCP-1 and IL-8 secretion were increased after stimulation with Hcy. It showed that the monocytes were as major cellular source to Hcy-induced MCP-1 and IL-8 synthesis in cultured whole blood. The data was similar with the previously studied that the mono/macrophages functioned as major resource cells in the production of MCP-1 and IL-8 in local plaque\[26-29\].

Our study showed that the enhancement of Hcy level in the cultured whole blood led to the upregulation of MPO concentration. MPO was as the marker of...
cardiovascular event, and also considered as the important regulator of vascular disease and deep venous thrombosis. Thus, increased MPO induced by Hcy could be one of the mechanisms by which hyperhomocysteinemia induced the initiation and progression of vascular disease. Because IL-8 was the powerful promoter of MPO release, meanwhile, we found Hcy could not promote the release of MPO from the neutrophils directly (data no shown). Thus, the Hcy-induced IL-8 secretion from the monocytes in the cultured whole blood might play the vital role in the upregulation of MPO level. However, more studies about this issue are needed to get more decisive conclusion.

Previous work suggested that cytokines such as IL-1β and TNF-α could regulate IL-8 and MCP-1 production. However, our initial study found that Hcy did not enhance the secretion of TNF-α and IL-1β in cultured human whole blood (data not shown). It suggested that IL-1β and TNF-α were not involved in Hcy-induced expression of MCP-1 and IL-8 in cultured human whole blood. Therefore, the mechanism of Hcy-induced MCP-1 and IL-8 secretion was different from that of IL-1β and TNF-α.

Although as an ex vivo model, cultured whole blood may have more considerable relevance with respect to circulation level than isolated cultured cells, additional clinic investigation about hyperhomocysteinemia patients are still needed before the final conclusion can be addressed.

In conclusion, Hcy induced MCP-1 and IL-8 secretion in cultured human whole blood, especially in monocytes via oxidative stress mechanism. These results had several clinical implications. First, the experiment was performed at the cultured whole blood, not in the single isolated cells. Compared with the previous studies, it was more helpful to understand the influence of hyperhomocysteinemia. For example, we found Hcy could trigger MCP-1 and IL-8 production even at 10 µmol/L. This finding was more direct to answer the question whether Hcy enhancement (hyperhomocysteinemia) in the plasma was the marker, or the important causation of vascular diseases. Second, the upregulation of MPO revealed the possible new mechanism in Hcy-induced progression of cardiovascular diseases. Finally, the intracellular ROS, particular the OH• radicals, played extremely important role in the Hcy-induced MCP-1 and IL-8 production. It suggested the anti-oxidative therapy would be considered to treat the hyperhomocysteinemia-related diseases.

**REFERENCES**

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