Homocysteine stimulates the expression of monocyte chemoattractant protein-1 receptor (CCR2) in human monocytes: possible involvement of oxygen free radicals

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INTRODUCTION

Atherosclerosis is a principal contributor to the pathogenesis of myocardial and cerebral infarction. Homocysteinaemia is an important risk factor for atherosclerosis [1–5]. Abnormal levels of homocysteine of up to 0.1–0.25 mM in blood have been reported in patients with homocysteinaemia [6–8]. A significant proportion (up to 30–40%) of patients with coronary artery disease displayed elevated homocysteine levels [9]. Plausible mechanisms of homocysteine-induced atherosclerosis include endothelial dysfunction, increased proliferation of smooth-muscle cells, promotion of lipoprotein oxidation and platelet activation, enhanced coagulability, increased cholesterol synthesis in hepatocytes and enhanced expression of monocyte chemoattractant protein 1 (MCP-1) in vascular cells [4,8,10–12].

One of the important features of atherosclerosis is monocyte infiltration into the injured arterial wall followed by monocyte differentiation into macrophages. These macrophages then take up large amounts of lipids to become foam cells [13–15]. The recruitment of monocytes into the intima of the arterial wall can be induced by a chemokine, namely MCP-1 [16–20]. We reported recently that homocysteine stimulates MCP-1 expression in cultured endothelial cells [21] as well as in vascular smooth-muscle cells (VSMCs) [12]. In endothelial cells, homocysteine stimulates MCP-1 expression via the activation of the p38 mitogen-activated protein kinase pathway, leading to enhanced chemotactic response of homocysteine-treated monocytes. Further investigation revealed that the levels of superoxide were significantly elevated in cells incubated with homocysteine for 12–48 h. The addition of superoxide dismutase, a scavenger of superoxide, to the culture medium abolished the stimulatory effect of homocysteine on MCP-1 expression as well as the binding activity of the receptor. The stimulatory effect of homocysteine on the expression of CCR2 mRNA and the levels of CCR2 protein was also observed in human peripheral blood monocytes. In conclusion, the present study has clearly demonstrated that homocysteine stimulates CCR2 expression in monocytes, leading to an enhanced binding activity and chemotactic response. Homocysteine-induced superoxide formation might serve as one of the underlying mechanisms for this effect.

Key words: atherosclerosis, chemotaxis, oxidative stress, superoxide.
(genetically modified to develop atherosclerosis) that also lacked CCR2 [27]. An increased expression of the gene for CCR2 was reported in patients with hypercholesterolaemia [28]. High plasma concentrations of low-density lipoprotein significantly increased monocyte CCR2 expression and the chemotactic response to MCP-1 [28]. In contrast, it was reported by the same research group that oxidized low-density lipoprotein decreased monocyte CCR2 expression via peroxisome proliferator-activated receptor γ [29]. It was suggested that this negative regulation of CCR2 expression might promote the arrest of newly recruited monocytes in the arterial wall [29]. Lipopolysaccharide [30] and some cytokines such as tumour necrosis factor α [31] were also shown to inhibit CCR2 expression in human monocytes.

Although elevated concentrations of homocysteine are correlated positively with cardiovascular risk, the effect of homocysteine on monocyte CCR2 expression is poorly understood. In the present study we investigated the expression of CCR2 in homocytosteine-treated THP-1 cells (a human monocytic cell line) and human peripheral blood monocytes. Our results indicated that CCR2 was progressively up-regulated when monocytes were incubated with various concentrations of homocysteine. This effect might be mediated by the formation of superoxide (O$_2^-$) in homocysteine-treated cells.

EXPERIMENTAL

Cell culture

THP-1 cells, a human monocytic cell line, were purchased from A.T.C.C. (Manassas, VA, U.S.A.). Cells were cultured in RPMI 1640 medium containing 10% (v/v) low-endotoxin fetal bovine serum, 100 i.u./ml penicillin and 10 µg/ml streptomycin. For experiments, cells were cultured in the RPMI 1640 medium and the cell density was maintained at 5 x 10⁷ cells/ml to prevent cell differentiation [29]. Human monocytes were isolated from peripheral blood, collected from healthy volunteers, by the Ficoll-Paque density gradient centrifugation procedure (Amersham Pharmacia Biotech). Mononuclear cells were separated from platelets by four washes at 300 g for 10 min. Finally, monocytes were added to a culture dish containing 10% (v/v) FBS and left to adhere to the surface of the dish. After incubation for 45 min, non-adherent lymphocytes were removed from the culture dish. Adherent monocytes were recovered by incubation with PBS containing 10 mM EDTA for 5 min. After being washed in PBS, monocytes were resuspended in RPMI 1640 and used for experiments.

Analysis of CCR2 mRNA by nuclease protection assay

Total RNA was isolated from cultured cells with TriZol Reagent (Life Technologies). A nuclease protection assay was performed with a kit (Ambion, Austin, TX, U.S.A.) in accordance with the manufacturer’s instructions. In brief, total RNA (10 µg) was hybridized overnight at 30 °C with a 32P-end-labelled CCR2 oligonucleotide probe (Clontech, Palo Alto, CA, U.S.A.) followed by nuclease digestion to remove non-hybridized probe. After digestion, the protected fragments were resolved on a denaturing 12% (w/v) polyacrylamide gel containing 8 M urea. A positive control, 28 S rRNA oligonucleotide probe (Ambion), was used as an internal control. Bands corresponding to CCR2 mRNA and 28 S rRNA were detected by autoradiography and analysed with a gel documentation system (Bio-Rad Gel Doc1000). The ratio of CCR2 to 28 S rRNA was calculated and values were expressed as percentages of control.

Determination of CCR2 protein by Western immunoblotting analysis

The amount of CCR2 protein synthesized by THP-1 cells was determined by Western immunoblotting analysis. In brief, cellular proteins (40 µg) from control or homocysteine-treated cells were separated by electrophoresis by SDS/PAGE [12% (w/v) gel]. Proteins on the gel were then transferred to a nitrocellulose membrane. The CCR2 protein band was identified by polyclonal anti-CCR2B antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), which recognized 38 kDa human CCR2. Horseradish-peroxidase-conjugated secondary antibodies were used to develop the membrane. The protein bands were detected with enhanced chemiluminescence reagent (Amersham Pharmacia, Little Chalfont, Bucks., U.K.) and analysed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst® version 1.1).

Flow cytometry

The binding of MCP-1 to CCR2 on the surfaces of THP-1 cells was measured with Fluorokine human MCP-1 biotin conjugate Cytokine Flow Cytometry Reagent (R&D Systems, Minneapolis, MN, U.S.A.) [30,31]. In brief, cells were incubated for 24 h in the presence or absence of homocysteine. After being washed three times with PBS, the control or homocysteine-treated cells were incubated for 1 h with the biotinylated MCP-1. The cells were then incubated with avidin–FITC as recommended by the manufacturer (R&D Systems) and analysed with a Coulter EPICS Elite ESP Flow Cytometer (Beckman Coulter, Fullerton, CA, U.S.A.). For each determination, 10000 cells were analysed.

Chemotaxis assay

Monocyte chemotaxis was measured with a 48-well Micro Chemotaxis Chamber (Neuro Probe, Gaithersburg, MD, U.S.A.) [32]. THP-1 cells were incubated for 24 h in the presence or absence of homocysteine at various concentrations. After incubation, THP-1 cells were resuspended at 1.5 x 10⁶ cells/ml and transferred to the upper chamber of the Micro Chemotaxis Chamber. The chemoattractant, MCP-1 (0.1 µg/ml), was added to the lower chamber. The lower and upper chambers were separated by a polycarbonate membrane (5 µm pore size). The homocysteine-treated monocytes were left to transmigrate for 1 h. After transmigration, the surface of the membrane facing the THP-1 cell suspension was scraped and washed three times in accordance with the manufacturer’s instructions. The cells that had migrated towards the chemoattractants were fixed and then stained with haematoxylin. The number of migrated monocytes was determined by counting the cells in five high-power fields under light microscopy.

Determination of cellular O$_2^-$ content

The content of O$_2^-$ radical anions was determined by the Nitro Blue Tetrazolium (NBT) reduction assay [33]. After incubation for 24 h in the absence or presence of homocysteine, cells were washed three times with Hanks balanced salt solution and then incubated for a further 2 h in Krebs–Henseleit buffer containing 1 mg/ml NBT. At the end of the incubation, cells were washed three times with Hanks balanced salt solution and lysed with 5% (w/v) SDS/80 mM phosphate buffer (pH 7.8) followed by centrifugation for 5 min at 13000 g. The absorbance of the supernatant
at 540 and 450 nm was determined as described previously [33]. Formazan was generated by the reduction of NBT, which was correlated with the amount of $O_2^-$ formed in cultured cells.

**Statistical analysis**

The results were analysed with a two-tailed independent Student’s $t$ test. The level of statistical significance was set at $P < 0.05$.

**RESULTS**

**Effect of homocysteine on the expression of CCR2 mRNA**

THP-1 cells were first incubated with homocysteine (0.1 mM) for 6, 12, 24 and 48 h. After incubation, CCR2 mRNA was determined by nuclease protection assay. As shown in Figure 1(A), THP-1 cells expressed basal levels of CCR2 mRNA before treatment with homocysteine (0 h of incubation). However, homocysteine treatment resulted in a significant increase in CCR2 mRNA expression at 12 h of incubation and reached a maximum at 24 h (Figure 1A). This stimulatory effect of homocysteine on CCR2 mRNA expression occurred in a concentration-dependent manner (0.05–0.2 mM) (Figure 1B). To determine whether homocysteine could prolong the stability of CCR2 mRNA, THP-1 cells were incubated with actinomycin D, a transcriptional inhibitor, in the absence or presence of homocysteine. As shown in Figure 2, the relative rate of CCR2 mRNA decay remained the same in homocysteine-treated cells as in control cells. It therefore seems that homocysteine could induce CCR2 expression at a transcriptional level.

**Effect of homocysteine on the expression of CCR2 protein**

The amount of CCR2 protein in cultured THP-1 cells was determined by Western immunoblotting analysis. In accordance with the expression of CCR2 mRNA, treatment with homocysteine significantly increased the amount of CCR2 protein in monocytes (Figure 3). The largest amount of CCR2 protein (183% of the control) was observed after cells had been incubated with homocysteine for 24 h (Figure 3). Similar experiments were performed with freshly isolated human blood monocytes. As shown in Figure 4, the expression of CCR2 mRNA and the levels of CCR2 proteins were elevated in those cells treated with homocysteine (0.05–0.2 mM) in the same manner as that observed in THP-1 cells. For the rest of the study, experiments were performed in THP-1 cells.

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![Figure 1](https://example.com/figure1.png)

**Figure 1** Expression of CCR2 mRNA in THP-1 cells

(A) Cells were incubated in the absence (control) or presence of homocysteine (0.1 mM) for various periods. After incubation, nuclease protection assays were performed to determine CCR2 mRNA expression. (B) Cells were incubated with 0.01–0.2 mM homocysteine (Hcy) for 24 h, after which a nuclease protection assay was performed. Values are expressed as the relative expression of CCR2 mRNA normalized to 28 S rRNA levels. Cells without homocysteine treatment were used as the control. Results are depicted as means ± S.D. (error bars) for five separate experiments. *$P < 0.05$ compared with control.
Figure 2 Effect of homocysteine on the stability of CCR2 mRNA in THP-1 cells

THP-1 cells were incubated in the absence or presence of 0.1 mM homocysteine for 24 h. Actinomycin D (at a final concentration of 5 μg/ml) was then added to the culture medium and cells were incubated for a further 2–8 h. The CCR2 mRNA levels were assessed by a nuclease protection assay. Results were expressed as a percentage of control (without addition of actinomycin D) and are depicted as means ± S.D. (error bars) for five separate experiments.

Figure 3 Effect of homocysteine on CCR2 protein levels in THP-1 cells

THP-1 cells were incubated in the absence or presence of homocysteine (0.1 mM) for various periods. After incubation, intracellular levels of CCR2 protein were determined by a Western immunoblotting analysis with anti-CCR2B antibodies. Results are expressed as means ± S.D. (error bars) for three separate experiments. *P < 0.05 compared with cells before treatment with homocysteine.

The binding of MCP-1 to CCR2 as a functional receptor on monocyte surface was examined by biotinylated MCP-1 binding assay. As shown in Figure 5, the binding of MCP-1 to monocytes was significantly elevated after incubation with homocysteine for 24 h (0.05–0.2 mM). The functional relevance of the increased surface expression of CCR2 protein in monocytes was examined further with a chemotaxis assay. Cells were incubated with homocysteine (0.01–0.2 mM) for 24 h. After incubation, the chemotactic response of homocysteine-treated monocytes to MCP-1 was examined. As shown in Figure 6, the number of homocysteine-treated monocytes that migrated towards the medium containing MCP-1 was significantly elevated in a concentration-dependent manner.

The effect of cysteine on the expression of CCR2 mRNA and protein was also examined. Treatment with cysteine (0.1 mM) did not affect the expression of CCR2 mRNA (Figure 7A) or the levels of CCR2 proteins in THP-1 cells (Figure 7B). Furthermore, cysteine treatment did not stimulate the binding of MCP-1 to THP-1 cells (Figure 7C).

Effect of homocysteine on cellular formation of \( \text{O}_2^- \)

To examine whether homocysteine-induced CCR2 expression involved oxidative stress, the effect of homocysteine on the formation of \( \text{O}_2^- \) in monocytes was examined. Cells were
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Figure 5 Effect of homocysteine on the binding activity of cell-surface CCR2

THP-1 cells were incubated for 24 h in the absence (control) or presence of homocysteine (0.01–0.2 mM). The binding of biotinylated MCP-1 to monocyte-surface CCR2 was determined by flow cytometry. (A) Representative FACS histograms of the binding of biotinylated MCP-1 to control and homocysteine-treated cells. (B) The results from (A) expressed as percentages of control and depicted as means ± S.D. (error bars) for three separate experiments. * $P < 0.05$ compared with control.

Figure 6 Chemotactic activity of THP-1 cells

Cells were incubated for 24 h in the absence or presence of homocysteine (0.01–0.2 mM). The MCP-1-induced chemotactic activity was determined by using a 48-well Micron Chemotaxis Chamber. The results are expressed as numbers of migrated monocytes per high-power field (HPF) under light microscopy and are shown as means ± S.D. (error bars) for three separate experiments. * $P < 0.05$ compared with control.

Figure 7 Effect of cysteine on the expression of CCR2 mRNA (A), levels of CCR2 protein (B) and the binding activity of cell-surface CCR2 (C)

THP-1 cells were incubated in the absence or presence of cysteine (0.1 mM) or homocysteine (0.1 mM) for 24 h. (A, B) After incubation, a nuclease protection assay was performed to determine the expression of CCR2 mRNA (A) and a Western immunoblotting analysis was performed to determine the levels of CCR2 protein (B). (C) Representative FACS histograms of binding of biotinylated MCP-1 to control and homocysteine-treated or cysteine-treated cells.

Figure 8 $O_2^\cdot$ levels in THP-1 cells

Upper panel: cells were incubated with homocysteine (0.1 mM) for various periods. Lower panel: cells were incubated for 24 h in the absence or presence of various quantities of homocysteine. In both experiments the formation of cellular $O_2^\cdot$ was determined by the NBT reduction assay and is expressed as the percentage of formazan formation in control cells. Results are shown as means ± S.D. (error bars) for three separate experiments. * $P < 0.05$ compared with control.
To investigate whether a decrease in cellular $O_2^-$ levels could prevent homocysteine-induced CCR2 expression, a $O_2^-$ scavenger, SOD, was added to the culture medium. Monocytes were incubated for 24 h with homocysteine (0.1 mM) in the absence or presence of SOD. First we determined the effect of SOD on cellular $O_2^-$ formation. As shown in Figure 9(A), the addition of SOD abolished the stimulatory effect of homocysteine on the levels of $O_2^-$ in those cells. Next we analysed the expression of CCR2 mRNA and CCR2 protein. Treatment with SOD also abolished the stimulatory effect of homocysteine on CCR2 mRNA expression (Figure 9B) as well as that on CCR2 protein expression (Figure 9C). Finally, the effect of SOD on the binding of MCP-1 to CCR2 as a functional receptor on monocyte surface was determined by flow cytometry. As shown in Figure 9(D), the addition of SOD to the culture medium reversed the stimulatory effect of homocysteine on the binding activity of monocyte CCR2 to MCP-1. This treatment did not affect the basal levels of $O_2^-$ formation, the expression of CCR2 or the binding activity of CCR2 in control cells (Figure 9). Taken together, these results suggest that homocysteine-induced $O_2^-$ formation might be one of the mechanisms causing enhanced CCR2 expression in monocytes.

**DISCUSSION**

The present study clearly demonstrates that (1) treatment with homocysteine stimulates the expression of CCR2 mRNA accompanied by an increased expression of CCR2 protein in THP-1 cells and human blood monocytes leading to enhanced binding activity and chemotactic response in monocytes; (2) this stimulatory effect is mediated by an overproduction of $O_2^-$ radical anions in homocysteine-treated cells. These results suggest that homocysteine-induced CCR2 expression in monocytes might facilitate the recruitment of monocytes into the arterial wall, where those cells differentiate into macrophages. Macrophages are capable of producing various cytokines and growth factors that can, in turn, contribute to the development of atherosclerosis.

Recent evidence suggests that oxidative stress is involved in homocysteine-mediated cardiovascular risk [34-37]. The homocysteine-induced production of free radicals has been indicated as one of the mechanisms causing cell damage in the vascular wall [38,39]. SOD, a $O_2^-$ scavenger, might be able to alleviate the cytotoxic effect of homocysteine caused by the cellular formation of $O_2^-$. [36-39]. In our previous study, SOD was shown to partly reverse the stimulatory effect of homocysteine on NF-$\kappa$B activation as well as on MCP-1 expression in VSMCs [12]. Our results suggested that homocysteine might increase $O_2^-$ production in VSMCs, which in turn could contribute to an increased degradation of inhibitory $\kappa$B-$\alpha$ and the activation of NF-$\kappa$B. In the present study we investigated a potential mechanism of homocysteine-stimulated expression of CCR2 in monocytes. Our results suggest that homocysteine-induced $O_2^-$ formation might contribute to the up-regulation of CCR2 expression in monocytes. The addition of SOD can abolish the effect of $O_2^-$ production induced by homocysteine. Recently, Upchurch et al. [34] reported that homocysteine stimulated the production of reactive oxygen species and impaired the glutathione peroxidase-mediated detoxification of $H_2O_2$ in bovine aortic endothelial cells, leading to a decrease in bioavailable nitric oxide. Wilcken et al. [37] reported that the levels of circulating SOD was correlated with the levels of plasma homocysteine in homocysteinemic patients. Their results suggested that an elevation of extracellular SOD might represent a protective antioxidative response to homocysteine-induced oxidative changes [37]. Homocysteine contains a reactive thiol group that can undergo oxidation to produce reactive oxygen species including $O_2^-$. [38,40]. Although the precise mechanism of homocysteine-mediated atherosclerosis is not clear, oxidative stress due to the overproduction of $O_2^-$ has been proposed to have a role in homocysteine-induced endothelial cell injury.

**Effect of superoxide dismutase (SOD) on cellular levels of $O_2^-$ and CCR2 expression in monocytes**

To investigate whether a decrease in cellular $O_2^-$ levels could prevent homocysteine-induced CCR2 expression, a $O_2^-$ scavenger, SOD, was added to the culture medium. Monocytes were incubated for 24 h with homocysteine (0.1 mM) in the absence or presence of SOD. First we determined the effect of SOD on cellular $O_2^-$ formation.

![Figure 9](image-url)

**Figure 9** Effect of SOD on cellular $O_2^-$ formation (A), CCR2 expression (B, C) and binding activity of cell-surface CCR2 (D)

THP-1 monocytes were incubated for 24 h in the absence (control) or presence of homocysteine (0.1 mM). In some experiments, SOD (300 units/ml) was included in the culture medium. After incubation, the cellular formation of $O_2^-$ was determined by NBT reduction assay (A), CCR2 mRNA expression was determined by nuclear protection assay (B), CCR2 protein level was determined by Western immunoblotting analysis (C) and the binding activity of cell-surface CCR2 was measured by flow cytometry (D). The results are expressed as percentages of control and are shown as means ± S.D. (error bars) for three separate experiments. *P < 0.05 compared with control.
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[3,39–41]. The relevance of the proposed pro-oxidant effect of homocysteine has recently been challenged by Jacobsen [40], in that it is necessary to identify the oxidative stress markers in homocysteinaemic subjects with cardiovascular disease by using more sensitive techniques to support the notion that oxidative stress due to homocysteinaemia has a role in vascular injury. In the present study, treatment with homocysteine caused a significant elevation of cellular O$_2^-$ as well as an increase in the expression of CCR2. The addition of SOD not only prevented the elevation of cellular O$_2^-$ levels induced by homocysteine but also alleviated homocysteine-stimulated CCR2 expression as well as the homocysteine-enhanced binding activity of monocyte surface CCR2 to MCP-1. These results suggest that the overproduction of O$_2^-$ might be involved in homocysteine-stimulated CCR2 expression in monocytes. A recent study [42] revealed that H$_2$O$_2$ and the GSH-depleting drug buthionine sulfoximine stimulated CCR2 expression in human monocytes. Furthermore, treatment with antioxidants such as pyrrolidine dithiocarbamate could decrease the expression of CCR2 and other chemokine receptors in those cells, indicating a positive effect of oxidative stress on CCR2 expression [42]. Although results from several studies indicate that SOD might have a role in homocysteine-induced vascular cell dysfunction [12,34,37], the causative effect of homocysteine on the activity of this enzyme remains to be investigated.

The normal range of fasting plasma homocysteine in adults is usually 5–15 μM [43–45]. Homocysteinaemia or hyperhomocysteinaemia is defined as a plasma homocysteine concentration of more than 15 μM and hyperhomocysteinaemia is classed as moderate (15–30 μM), intermediate (30–100 μM) and severe (more than 100 μM) [44,45]. Epidemiological evidence indicates that even moderate or intermediate homocysteinaemia is associated with an increase in the risk for atherosclerosis [5,46]. In the present study, homocysteine at a concentration of 50 μM significantly stimulated the expression and the activity of CCR2 in THP-1 cells as well as in freshly isolated human blood monocytes. However, it remains to be investigated whether this stimulatory effect occurs in the vascular wall of patients with moderate or intermediate elevations in plasma homocysteine levels.

The advantages of using THP-1 as monocyte model in our study were twofold. First, THP-1 monocytes possess human monocyte-like characteristics [47,48]. Many investigators have used monocytes to study the expression of MCP-1 and chemotactic activity [28,49,50]. For example, oxidized low-density lipoprotein/lipopolysaccharide have been shown to stimulate THP-1 cell chemotaxis via the activation of p38 mitogen-activated protein kinase [51]. Han et al. [28] reported that low-density lipoprotein could enhance MCP-1-mediated chemotaxis in THP-1 cells. The second advantage of using THP-1 is that the homogeneity of this cell line permits the comparison of findings obtained from different experiments.

In conclusion, the present study has demonstrated for the first time that homocysteine stimulates the expression of the MCP-1 receptor CCR2 in THP-1 cells and in human peripheral blood monocytes. Such stimulatory effect was mediated by oxidative stress, namely an increase in cellular O$_2^-$ levels. Homocysteine-induced MCP-1 expression in vascular cells, together with enhanced CCR2 expression in monocytes, might represent a mechanism of homocysteine’s causing enhanced monocyte infiltration into the arterial wall during atherogenesis.

We thank Dr Y. L. Siow for his valuable suggestions, Ms Y. H. Chung for her technical assistance, and Dr N. S. Wong for the use of the flow cytometer in the Department of Biochemistry. This study was supported by a grant (HKU7346/00M) from the Research Grant Council of Hong Kong, Special Administration Region, China, to K.O.

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Received 5 January 2001; March 2001; accepted 23 March 2001