Inflammatory markers, vascular disease and homocysteine

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Publication date
1999

Citation for published version (APA):

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Download date:15 Aug 2023
ELEVATED LEVELS OF HOMOCYSTEINE INCREASE IL-6 PRODUCTION IN MONOCYTIC MONO MAC 6 CELLS

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Submitted
SUMMARY

Hyperhomocysteinemia is a risk factor for atherosclerosis and thrombosis. The aim of this study was to analyze if exposure of monocytic cells to increased levels of homocysteine (HCY) induces the accumulation of inflammatory mediators. IL-6 production by monocytic cell line Mono Mac 6 (MM6) was 1.7 fold increased in the presence of 50 μmol/L HCY and 3.5 fold with 200 μmol/L HCY. Incubation with homocystine resulted in a comparable dose-dependent increase, but neither cysteine nor methionine stimulated IL-6 accumulation. Elevated homocysteine concentrations did not affect the production of IL-8 and MCP-1 in MM6. Furthermore, LPS stimulation of MM6, cultured with elevated HCY (200 μmol/L) levels, resulted in a 3.5 fold increased response after 18 hours whereas no effect on LPS-induced IL-8 and MCP-1 response was observed. In conclusion, increased concentrations of homocysteine induce IL-6 accumulation in monocytic cells. After treatment with homocysteine, monocytic cells become more susceptible to endotoxin. This study is in favor of an association between homocysteine and monocytic IL-6 production.
INTRODUCTION

Elevated plasma levels of HCY are generally considered to be a risk factor for atherosclerosis and venous thrombosis (reviewed by 1 to 3). Whether HCY is the cause or an innocent bystander of vascular disease is still questionable. Genetic studies show data that are in favor for the innocent bystander theory. Mutations in genes responsible for homocysteine-catabolizing enzymes show an association with moderate increased plasma concentrations of HCY, but do not increase the risk for cardiovascular disease.4,5 On the other hand, individuals homozygous for cystathionine β-synthase deficiency, who have severely increased homocysteine plasma concentrations, suffer from premature vascular disease, suggesting that elevation of the homocysteine level is causing vascular damage.6 Furthermore, many in vitro studies have attributed a wide range of effects to homocysteine, including damage to endothelial cells, elevated procoagulant activity, increased collagen synthesis, and enhanced proliferation of smooth muscle cells.7,8 However, most of these effects occurred at concentrations of homocysteine (1 to 10 mmol/L), in its reduced form, that were highly non-physiological.9

Recently, several studies have shown that inflammatory processes play an important role in the pathogenesis of vascular disease.10 Damage to intravascular cells may lead to increased cytokine and chemokine production and thus a chronic inflammation. This chronic inflammatory response may be accompanied by procoagulant activity, chemotaxis, and acute phase response, thereby contributing to initiation and development of thrombosis and atherosclerosis.11,12

So far no reports are known on the association between hyperhomocysteinemia and cytokine production by monocytes. In this study we report on the analysis of the IL-6, IL-8 and MCP-1 production by monocytes cultured under conditions with modestly elevated HCY concentrations and on the effects of HCY on the inflammatory response to LPS. For these analyses the human monocytic cell line Mono Mac 6 (MM6) was used as a model, as this cell line exhibits characteristics of mature blood monocytes.13 We show that elevated concentrations of HCY result in an increased IL-6 production and an elevated IL-6 response after LPS stimulation.

MATERIALS AND METHODS

L-Homocysteine thiolactone, DL-homocysteine, L-cysteine, L-homocystine, L-methionine, 5.5'-dithio-(bis-2-nitrobenzoic acid (DTNB or Ellman's reagent), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co.(St. Louis, MO). RPMI 1640 and Iscove's modified medium (IMDM) (both media were purchased from Biowhittaker, Bioproducts, Heidelberg, Germany) as well as foetal calf serum (Integro B.V., Zaandam, the Netherlands) and homocysteine and control components were endotoxin free as
proven with the Limulus Amebocyte Lysate (LAL) assay.\textsuperscript{14} Lipopolysaccharide (LPS) from 
\textit{Escherichia Coli} 0111:B4 (Difco Laboratories, Detroit, MI) was vortexed for 30 minutes 
before use.

\textit{Mono Mac 6 cell culture}

The pre-monocytic cell line Mono Mac 6 (MM6) was used in this study as these cells exhibit 
characteristics of mature monocytes, including phagocytosis, CD14 expression, adhesion to 
endothelial cells and cytokine production.\textsuperscript{13,15,16} Mono Mac 6 (MM6) was kindly provided by 
Dr. H.W.L. Ziegler-Heitbrock and was cultured in IMDM supplemented with 10% foetal calf 
serum (FCS). Before addition of the test medium the cells were cultured for three days in 
RPMI 1640 with 10% FCS at a concentration of 0.5 x10\textsuperscript{6} cells/ml. The monocytic 
characteristic of the MM6 cell line was routinely screened by FACS analysis of the CD14 
expression.

\textit{Preparation of homocysteine and control culture media}

L-homocysteine (HCY) was prepared following the procedure of Duerre and Miller, by 
solubilizing L-homocysteine thiolactone in 5 mol/L NaOH, 2.5 mol/L HCl and 0.5 mol/L phosphate buffer p\textsubscript{H} 7.4 to a stock concentration of 0.3 mol/L HCY.\textsuperscript{17} The solution was 
filtered through a 0.2 \textmu m filter (Schleicher & Schnell, Dassel, Germany). Conditioned medium 
was prepared by diluting HCY or one of the control compounds in RPMI 1640 with 10% FCS 
after which the medium was pre-incubated at 37°C in a CO\textsubscript{2} incubator for 24 hours. The free 
SH-group content was determined using Ellman’s reagent (0.1 mmol/L DTNB diluted in 0.1 
 mol/L Tris pH 8.1) and measured spectrophotometrically at 405 nm.\textsuperscript{18} No free SH-groups 
could be detected after the pre-incubation. Conditioned medium, to which DL-homocysteine, 
L-cysteine, L-homocystine or L-methionine was added, was prepared parallel to the HCY 
medium. Homocystine is the dimer of HCY, therefore it was used at half the concentration of 
HCY.

MM6 was incubated at a concentration of 1x10\textsuperscript{6} cells/ml for 3 days in the conditioned 
medium in a 6 wells plate (Costar, Cambridge, USA), after which the cell concentration was 
counted in a Bürker counting chamber. The viability of the cells was tested using the MTT 
cytotoxicity test.\textsuperscript{19} After pre-incubation with HCY or one of the controls, the cells were washed and stimulated 
with 1.0 \mu g/ml LPS for 1 to 18 hours at 37°C in a 24 wells plate (Costar). The cell suspension 
was centrifuged and the supernatant was stored at -20°C until analysis.

\textit{Analysis of IL-6, IL-8 and MCP-1}

IL-6 and IL-8 were analyzed in the supernatant of the cell culture experiments using 
commercially available ELISAs (Central Laboratory of the Dutch Red Cross Blood 
Transfusion Service (CLB), Amsterdam, The Netherlands). MCP-1 levels were analyzed using
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rat anti human MCP-1 as a coating antibody, Escherichia coli MCP-1 as the standard, and biotinylated rat anti human MCP-1 as the second antibody (all MCP-1 reagents were from Pharmingen, San Diego, CA). The detection limit of the ELISAs was 1.2 pg/ml for IL-6, 2.0 pg/ml for IL-8 and 30 pg/ml for MCP-1.

**Statistical analysis**

At least six separate experiments were performed in triplicate. The increase of cytokine and chemokine production, which was corrected per 10^6 cells, was calculated as the percentage of the production compared to the control condition of each experiment separately. Comparison of the percentages of cytokine and chemokine production was performed using the Mann Whitney U test for paired non-parametric values. P< 0.05 was considered significant.

**RESULTS**

The monocytic cell line Mono Mac 6 was cultured for 3 days in conditioned medium with homocysteine, homocystine or one of the control compounds. The ratio of increased cytokine or chemokine production under the homocysteine conditions was calculated for every experiment separately in comparison to the control conditions. Incubations for 3 days with 50 μmol/L respectively 200 μmol/L HCY lead to 1.73 (±0.21) and 3.52 (±0.42) fold increased IL-6 production (P=0.005 respectively P<0.001)(Fig. 1a). A similar increase in IL-6 production was detected after culturing with 100 μmol/L homocystine (4.25±0.86)(P=0.005) or 200 μmol/L DL-homocysteine (3.31±0.64)(P=0.005). No significant increase was observed when 10 μmol/L HCY (1.29±0.07), 200 μmol/L cysteine (0.67±0.07), or 200 μmol/L methionine (1.59±0.31) were used (Fig. 1a).

IL-8 and MCP-1 production were not significantly affected upon incubation of MM6 with HCY (or one of the controls) (Fig. 1b and 1c). When the viability of the MM6 cell line was analyzed using the MTT cytotoxicity test, no significant loss of cell viability after the three-day incubation was detected (Fig. 1d).

To evaluate the effect of homocysteine on the inflammatory response, the MM6 cells were treated with LPS. LPS stimulation of MM6 cells, cultured in the presence of 200 μmol/L homocysteine for 3 days, resulted in a 1.8 fold increased IL-6 response after 4 hours compared to cells cultured under control conditions, and a 4-fold increased response after 18 hours. The IL-6 production of MM6 cells which were preincubated with 200 μmol/L cysteine or 50 μmol/L HCY did not increase upon LPS stimulation (fig. 2a). The IL-8 or MCP-1 response of MM6 cells was not significantly increased by pre-treatment with HCY or cysteine (fig 2b, 2c).
Figure 1. Inflammatory mediator production under hyperhomocysteinemic culture conditions. IL-6 (A), IL-8 (B) and MCP-1 (C) production was analyzed in the supernatant of monocytic cell line MM6 after a 3 day culture in conditioned medium with different concentrations of L-homocysteine, L-cysteine, L-homocystine and L-methionine. The increase in production was calculated as the percentage of control in each separate experiment. The mean ± SEM of at least 6 experiments is depicted. Homocysteine is the dimer of homocysteine, therefore it was used at half of the concentration as is shown in the graph (respectively 5, 25 and 100µM). Furthermore the percentages of MTT incorporation is shown as measure of cytotoxicity of the culture conditions in figure 1D. *P=0.005, **P<0.001

--- Homocystine --- Cysteine
--- Homocystine --- Methionine
DISCUSSION

This study analyzed the in vitro association of elevated concentrations of HCY and the production of inflammatory mediators in monocytic cells. We found that HCY increased IL-6 production by Mono Mac 6 cells in a concentration dependent manner, while the IL-8 and MCP-1 production did not change. When MM6 cells were pretreated with HCY and subsequently stimulated by endotoxin, HCY-pretreated cells produced more IL-6. In contrast, incubation with HCY did not affect the endotoxin induced IL-8 or MCP-1 response. Although it is still not established that elevated concentrations of homocysteine induce vascular damage directly, our data suggest that elevated concentrations of HCY might contribute to vascular disease by directly inducing IL-6 production, or by augmenting the effects of other inflammatory stimuli.

How is increased IL-6 production related to atherosclerosis? First, IL-6 induces the acute phase response, thereby inducing the production of acute phase proteins such as CRP, fibrinogen and FVIII. Furthermore, IL-6 was shown, in vitro, to induce smooth muscle cell proliferation and adhesion of lymphocytes to vascular endothelium, and IL-6 contributes to coagulation activation by inducing the generation of thrombin.

In order to attenuate the effect of elevated HCY and to analyze the effect of HCY on the monocytes in combination with another stimulus, the response of HCY treated monocytes was analyzed after stimulation with endotoxin. MM6 cells pre-incubated with HCY reveal a four times increased IL-6 response after a LPS stimulation compared to cells which were pre-incubated under control conditions, while the IL-8 and MCP-1 response remained unchanged. Hence, MM6 cells are more susceptible to LPS when cultured under hyperhomocysteinemic condition.
In this study the culture conditions were adjusted to reflect a physiological situation by using low concentrations of HCY and by pre-incubating the medium with serum, which reduces the percentage of free sulfur hydryl groups. Elevated concentrations of the dimer of HCY, homocystine, as well as of DL-HCY, induced the IL-6 production of MM6 in a similar manner as L-HCY derived from L-HCY thiolactone, whereas, control experiments with cysteine and methionine showed no increased IL-6 production. These results strongly suggest that the increased IL-6 production in MM6 results from an increased concentration of HCY or homocystine respectively and is not caused by the free SH-groups or the presence of thiolactone. Several in vitro studies have shown a toxic effect of HCY on cell viability and function, such as inhibition of the anticoagulant activity of the endothelium, deregulation of nitric oxide (NO), increased platelet adhesion and stimulation of smooth muscle cell growth. However, the in vivo relevance of these effects is questionable, as these studies were performed using high non-physiological HCY concentrations (1 to 10 mmol/L), while it has been shown that under physiological conditions HCY is present at low concentrations (15-50 \( \mu \)mol/L) and for only 5% in the reduced form. Furthermore, in most studies, the observed effect was not specific for HCY and could also be determined with other non-HCY related sulfur-containing compounds.

The question how elevated concentrations of homocysteine induce the IL-6 production of monocytic cells remains unanswered. We can only speculate that homocysteine might effect the IL-6 gene by a signal transduction mechanism through receptors for homocysteine that were described recently and that are coupled to phospholipase and diacylglycerol production. Future experimental work will be necessary to elucidate this mechanism.

In conclusion, elevated concentrations of HCY might contribute to vascular disease by increasing the production of monocytic IL-6, which in turn is able to induce the production of acute phase response, smooth muscle cell proliferation and coagulation activation.

REFERENCES
