Preliminary study of the genotoxic potential of homocysteine in human lymphocytes \textit{in vitro}

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Homocysteine (Hcy), an immediate precursor of methionine (Met), is considered a risk factor for cardiovascular disease, Alzheimer’s disease and neural tube defects. Hcy concentration is also reported to correlate positively with the micronucleus index in lymphocytes \textit{in vivo}, a marker of chromosome damage. However, it is unclear whether Hcy is genotoxic or simply a biomarker of folate deficiency, a known cause of chromosome damage. We investigated whether high concentrations of Hcy are genotoxic to human lymphocytes \textit{in vitro} using the cytokinesis-block micronucleus assay. Eighteen lymphocyte cultures were initiated in Met-free and serum-free RPMI 1640 medium for each of four male volunteers aged 22–23 years. At 0, 24, 44 and 72 h, cultures were spiked with L-Hcy or L-Met to achieve concentrations ranging between 50 and 400 $\mu$M. The concentration of Hcy at 96 h ranged from 19.45 ± 2.34 to 149.02 ± 28.16 $\mu$M in Hcy cultures and 0.91 ± 0.17 to 2.15 ± 0.9 $\mu$M in Met cultures spiked with 50 and 400 $\mu$M of metabolite, respectively. Forty-four hours after mitogen stimulation, cytokinesis was inhibited with cytochalasin B. After 96 h, cells were transferred to microscope slides and the frequency of micronucleated-binucleate and necrotic cells was scored. Neither Hcy ($P = 0.24$) nor Met ($P = 0.93$) had an apparent dose effect on micronucleus frequency. However, when data were pooled, micronuclear frequency was moderately higher (50.1%) in Hcy- than in Met-spiked cultures ($P = 0.04$; paired $t$-test). Hcy concentration was positively correlated with necrosis ($P < 0.0005$; $r^2 = 0.276$), however, when data were pooled, levels of necrosis were higher in Met- than in Hcy-spiked cultures ($P = 0.01$; paired $t$-test). Further research is required to define more clearly the genotoxic and cytotoxic potential of homocysteine and its metabolites.

Introduction

Homocysteine (Hcy) is the immediate precursor of the amino acid, methionine (Met; see Figure 1). In humans, blood concentrations of Hcy may become elevated as a result of deficiency in folate (Jacob \textit{et al.}, 1998; Kang \textit{et al.}, 1987), vitamin B$_6$ (Siri \textit{et al.}, 1998) or vitamin B$_{12}$ (Brattstrom \textit{et al.}, 1988). Furthermore, hyperhomocysteinemia may also be caused by mutations within the genes that encode methylene-tetrahydrofolate reductase (MTHFR) (Frost \textit{et al.}, 1995) and cystathionine $\beta$-synthase (Mudd \textit{et al.}, 1964). Hcy is of great interest because it has been identified as a putative risk factor for arteriosclerosis (McCuly, 1969), myocardial infarction (Verhoef \textit{et al.}, 1996), peripheral arterial occlusive disease (Malinow \textit{et al.}, 1989), subcortical vascular encephalopathy (Fassbender \textit{et al.}, 1999), Alzheimer’s disease (Clarke \textit{et al.}, 1998) and neural tube defects (Mills \textit{et al.}, 1995; van der Put \textit{et al.}, 1997).

Recently, the novel observation has been made that plasma Hcy concentrations positively correlate with baseline levels of genetic damage, as measured by the cytokinesis-block micronucleus assay, in humans (Fenech \textit{et al.}, 1997, 1998). Multiple regression analysis suggested that the effect of Hcy is independent of vitamin B$_{12}$ and folate (Fenech \textit{et al.}, 1998). This finding may be of importance because it is well established that levels of chromosome damage are significantly and positively correlated with cancer risk in humans (Hagmar \textit{et al.}, 1994, 1998; Bonassi \textit{et al.}, 2000).

Hcy is cytotoxic to cells in culture (Wall \textit{et al.}, 1980; Starkebaum and Harlan, 1986), and this may be one of the underlying causes of its postulated role in atherogenesis. However, the question remains as to whether Hcy is genotoxic to cells or whether it is simply a biomarker of some other event that is causing DNA damage, e.g. folate deficiency, which causes chromosome breakage and micronucleus formation (Titenko-Holland \textit{et al.}, 1998). Various reports show that high concentrations (100 $\mu$M to 10 mM) of Hcy facilitate the generation of hydrogen peroxide (H$_2$O$_2$) \textit{in vitro} (Wall \textit{et al.}, 1980; Starkebaum and Harlan, 1986; Stamler \textit{et al.}, 1993). It is thought that H$_2$O$_2$ is a product of the oxygen-dependent oxidation of Hcy (Starkebaum and Harlan, 1986). H$_2$O$_2$ is known to induce necrosis and micronuclei in human lymphocytes (Crott and Fenech, 1999).

Studies have also shown that Hcy promotes the expression of inducible nitric oxide synthase (iNOS) \textit{in vitro} and, thereby, the production of nitric oxide (NO) in a dose-dependent fashion (Upchurch \textit{et al.}, 1997; Welch \textit{et al.}, 1998; Ikeda \textit{et al.}, 1999). NO is known to inhibit methionine synthase, a key folate-metabolizing enzyme (Nicolaou \textit{et al.}, 1996). Inhibition of methionine synthase causes a maldistribution of folate derivatives, which is characterized by accumulation of 5-methyltetrahydrofolate (5-meTHF) (Horne and Holloway, 1997). The accumulation of folate in the 5-meTHF form (‘methyl folate trapping’) and subsequent reduction in the concentration of 5,10-methylenetetrahydrofolate (5,10-meTHF) promotes an increase in the ratio of dUMP to dTMP and thereby the misincorporation of uracil into DNA because 5,10-meTHF is the methyl group donor for the conversion of dUMP to dTMP (Blount and Ames, 1995). The excision of two opposing uracils, within 14 bp, has been shown to cause double-stranded DNA breaks (Dianov \textit{et al.}, 1991). Occupational exposure to another oxide of nitrogen, nitrous oxide (N$_2$O), has been reported to cause a four-fold elevation of micronucleus frequency in lymphocytes (Chang \textit{et al.}, 1996). N$_2$O is known to inactivate methionine synthase by oxidizing its cofactor, cobalamin (Drummond and Matthews, 1994).

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In an effort to clarify whether Hcy is a cause of chromosome damage or simply a biomarker of another chromosome-damaging event, we aimed to determine whether high concentrations (from high physiological to greater than physiological, i.e. 50–400 µM) of Hcy are genotoxic to human lymphocytes in folate- and vitamin B12-replete conditions *in vitro* by using the cytokinesis-block micronucleus (CBMN) assay. Cultures treated with Hcy were compared with control cultures treated with Met.

### Materials and methods

Fasted blood samples (36 ml, in lithium-heparinized evacuated containers) were collected from four male volunteers (aged 22–23 years) who consented to donate a blood sample. The study was approved by the Human Ethics Committee at CSIRO. Lymphocytes were isolated using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradients. Eighteen cultures (1 × 10⁶ cells/ml) were prepared for each volunteer in 1 ml of RPMI 1640 medium without Met, fetal bovine serum (FBS) or interleukin-2 (IL-2) (Trace Biosciences, Victoria, Australia) in 6 ml culture tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Serum-free conditions were used in order to give optimal control of Met and Hcy concentrations. In preliminary experiments (data not shown) we found that sufficient binucleated cell frequencies can be obtained in the CBMN assay with serum- and IL-2-free medium, but that the culture time needs to be extended by harvesting cells at 96 h, i.e. 52 h after the addition of cytochalasin B rather than 28 h. Tubes were then gently spun and 100 µl of supernatant was removed and stored at −20°C for the measurement of d,L-Hcy concentrations. One hundred µl of medium containing 0, 1, 2, 4 or 8 mM d,L-Hcy or 0.5, 1, 2 or 4 mM L-Met (for comparison) was then added to the cultures to achieve concentrations ranging from 0 to 800 µM in the cell culture (0–400 µM of the active l-isomer). Cultures were prepared in duplicate. After sampling and spiking cultures with Hcy/Met, phytohaemagglutinin (PHA, 22 µg/ml) (Murex Biotech, Kent, UK) was added to stimulate mitogenesis. Cultures were then placed in a humidified incubator set at 37°C with 5% CO₂. The process of sampling and then spiking cultures was repeated at 24, 44 and 72 h after the addition of PHA. Forty-four h after the addition of PHA, cytochalasin B (4.5 µg/ml; Sigma, St Louis, MO, USA) was added (immediately after spiking) to inhibit cytokinesis. Ninety-six hours after addition of PHA, cultures were sampled for the final time before cells were transferred to microscope slides using a cytocentrifuge (Shandon Southern Products Ltd, Cheshire, UK), air-dried and stained using Diff-Quik (Lab Aids, NSW, Australia). The precise sequence of the above events is illustrated in Figure 2. Coded slides were scored by one scorer using established criteria (Fenech, 1993) with necrotic cells being counted as described by Crott and Fenech (1999) and Fenech *et al.* (1999). A total of 1000 binucleated cells was counted per slide/treatment for the micronucleus index and 500 for all other indices. The concentration of Hcy in cell culture medium was measured by high performance liquid chromatography (HPLC) with fluorescent detection (Shimadzu series 10A system controlled by Shimadzu class LC10, version 1 software) using the internal standard [mercaptoproprionyl glycerine (Sigma)] method of Vester and Rasmussen (1991). Hcy was derivatized with 7-fluorobenzofurazane-4-sulfonic acid (Fluka, Buchs, Switzerland). The HPLC system was equipped with a C18 reversed phase Microsorb column (25 cm × 4.6 mm, 5 µm particle size) (Varian, Walnut Creek, CA, USA) and an ODS2 guard column (SGE, Victoria, Australia).

Note that because l-Hcy is the metabolically active form, the relevant Hcy concentration is half of that of d,L-Hcy and comparisons with Met-treated cultures were made on that basis. Comparisons between different doses were made using repeated-measure analysis of variance (ANOVA) with Tukey’s post-test analysis and Kruskal–Wallis (with Dunn’s post-test) analyses. Comparisons between treatments (along with doses) were made using two-way ANOVA followed by individual one-tailed paired t-tests. Statistical calculations were performed using GraphPad Prism v.2.01 (GraphPad Inc., San Diego, CA, USA). Significance was accepted at *P* < 0.05.

### Results

As shown in Figure 3, Hcy accumulated over the culture period in both Hcy- and Met-treated cultures. At 96 h the mean concentration of Hcy in media ranged from 19.45 ± 2.34 (50 µM treatment) to 149.02 ± 28.16 µM (400 µM) and from 0.91 ± 0.17 (50 µM) to 2.15 ± 0.9 µM (400 µM) in the Hcy- and Met-spiked cultures, respectively. Note that these concentrations and those shown in Figure 3 are those immediately before spiking except for the final removal at 96 h. Therefore, with the exception of *t* = 0 values, the values give a measure of the amount of Hcy remaining in the culture medium –24 h after spiking with Hcy or Met.

Lymphocytes from two of the four volunteers exhibited a marked Met-dependency and hence the mitotic index was very low in Hcy-supplemented media (without Met). Met-dependency is the inability of cells to proliferate in media where Met has been replaced by its immediate precursor, Hcy. This made it impossible to score micronuclei at any Hcy concentration for one volunteer and for the two lowest Hcy doses for another. No cells were able to proliferate in the
The results of this experiment provide limited support for the hypothesis that high concentrations of Hcy increase the frequency of micronucleated lymphocytes compared with similar molar concentrations of Met in vitro. The unexpected Met-dependency of two of the four volunteers prevented the simultaneous absence of Hcy and Met and micronucleus data in once-divided cells could not be obtained.

There was no significant dose-related change in the micronucleus frequency of cultures spiked with Met ($P = 0.93$) or Hcy ($P = 0.24$) (Table 1). However, as shown in Figure 4A, the micronucleus frequency of Hcy-spiked cultures was moderately higher than that of cultures spiked with Met. Two-way ANOVA performed on the data yielded a $P$ value of 0.07 for the comparison between treatments and a $P$ value of 0.74 for the dose effect (in accordance with the one-way ANOVA results). Because there was no clear dose-response effect of Hcy or Met on micronucleus frequency, the data from all Hcy and Met cultures were pooled for comparison. The $P$ value of the paired t-test for this comparison was 0.04. These data suggest an increase in micronucleus frequency in Hcy-treated cultures compared with Met-treated cultures.

The addition of Hcy and Met significantly increased the proportion of binucleated cells compared with cultures without Hcy or Met (Table 1). In fact, Hcy concentration was positively correlated with the proportion of binucleated cells ($r = 0.07$). When the 0 µM Met/Hcy point was omitted from the analysis, a negative correlation between Met concentration and the proportion of binucleated cells approached significance ($P = 0.07$, $r^2 = 0.11$). The addition of 50–200 µM Met significantly increased necrosis compared with 0 µM Hcy/Met (Table 1). The addition of 400 µM l-Hcy significantly increased necrosis compared with 0 and 50 µM Hcy. Hcy concentrations were significantly and positively correlated with necrosis ($P < 0.0005$; $r^2 = 0.276$).

At the two lower spiking concentrations, more necrosis occurred in cultures treated with Met than with Hcy. Two-way ANOVA performed on the necrosis data yielded a $P$ value of 0.01 for the comparison between treatments and a $P$ value of 0.85 for the dose effect. $P$ values for the (paired $t$-test) comparison between Met and Hcy were 0.03, 0.04, 0.23 and 0.41 at 50, 100, 200 and 400 µM (l-isomer), respectively. Necrosis data for all Hcy and Met cultures were pooled into treatments and compared using a paired $t$-test. This analysis revealed that necrosis was significantly higher in cultures spiked with Met than with Hcy ($P = 0.01$).

Necrosis was significantly and positively correlated with the proportion of binucleated cells ($P < 0.0001$; $r^2 = 0.302$).

**Discussion**

The results of this experiment provide limited support for the hypothesis that high concentrations of Hcy increase the frequency of micronucleated lymphocytes compared with similar molar concentrations of Met in vitro. The unexpected Met-dependency of two of the four volunteers prevented the absence of Hcy and Met and micronucleus data in once-divided cells could not be obtained.

Table 1. Dose–responses in relation to l-homocysteine (Hcy) and methionine (Met) concentration. Effect on frequency of micronucleated (MNed), binucleated cells (BN) and necrosis.

<table>
<thead>
<tr>
<th>Spike (mM)</th>
<th>MNed BNs/1000 BNs</th>
<th>% BNs (of viable)</th>
<th>% Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Met</td>
<td>Hcy</td>
<td>Met</td>
</tr>
<tr>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>2.2±1.3</td>
</tr>
<tr>
<td>0.5</td>
<td>3.9±1.3</td>
<td>4.2±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.0±3.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0</td>
<td>2.8±0.4</td>
<td>4.9±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.7±2.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.0</td>
<td>2.7±0.5</td>
<td>5.9±1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.3±2.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.0</td>
<td>3.1±0.5</td>
<td>3.5±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.4±2.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>$P$ value</td>
<td>0.93</td>
<td>0.24</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data reported as mean ± SEM ($n = 4$). NA, insufficient binucleated cells to determine frequency of micronucleated binucleated cells. $P$ value is for dose effect. Kruskal-Wallis test used for micronucleated cell data and repeated-measures ANOVA used for percentage of binucleated cells and percentage necrosis.

<sup>a</sup>$n = 2$.

<sup>b</sup>Significantly different from 0 mM Met/Hcy ($P < 0.05$).

<sup>c</sup>Significantly different from 0.5 mM Hcy ($P < 0.05$).

<sup>d</sup>Significantly different from 0.5 mM Hcy/Met ($P < 0.05$).
scoring of micronuclei in some Hcy-spiked cultures, which reduced the potential statistical power of the study. More experiments are required to verify these observations in lymphocytes from a larger number of subjects whose lymphocytes are Met-independent as well as in those who are Met-dependent. It is evident that some Met will be required in the culture medium if the effects of Hcy on micronucleus expression in Met-dependent subjects are to be measured adequately.

Met-dependency is the inability of cells to proliferate in media where Met has been replaced by Hcy (Hoffman, 1982). It is associated, in some studies, with cancerous cells and even as a trait acquired during transformation (Hoffman, 1982; Stern et al., 1984). However, its relevance to cancer risk is unclear because it has been reported that in individuals with Met-dependent tumors this trait is also found in non-cancerous cells (Mikol and Lipkin, 1984). There is as yet no conclusive evidence that Met-dependence is a risk factor for cancer in humans. Furthermore, in a separate study we have shown that the Met-dependent phenotype is not associated with increased micronucleus frequency (Crott, J.W. et al., in press).

Previously it has been reported that plasma Hcy concentrations positively correlate with micronucleus frequency in humans (Fenech et al., 1997, 1998). Because the results presented here are preliminary, it would be premature to suggest a strong direct role of Hcy in micronucleus formation. It is not possible at this stage to exclude the possibility that the high plasma Hcy concentrations seen in people with high micronuclei counts (Fenech et al., 1997, 1998) may be a biomarker for folate or vitamin B12 deficiency, which promote chromosome damage, rather than being a cause of the damage. Indeed, negative correlations between plasma concentration of vitamin B12 and micronucleus frequency were also evident in the study groups that exhibited a positive correlation between Hcy and micronucleus levels (Fenech et al., 1998). It must be noted, however, that this experiment utilized a regime of acute exposure to Hcy in vitro. To obtain a definitive answer as to whether or not Hcy is genotoxic in vivo, further experiments testing the effects of artificially and chronically elevated plasma Hcy concentrations in laboratory animals under conditions of B vitamin repletion need to be conducted.

A potential confounding factor in these studies is the possibility that elevated Hcy concentrations could have increased S-adenosylmethionine levels considerably by increasing the rate of Met synthesis. Elevated S-adenosylmethionine concentrations would inhibit MTHFR (Kutzbach and Stokstad, 1971) and thereby divert folate towards the methylation of dUMP to form dTMP, which may, in turn, reduce micronuclear frequency and partially explain the absence of a clear dose–response effect for Hcy. Other possible explanations for the apparent lack of a dose–response effect for micronucleus frequency with Hcy concentration are: (i) the inability to obtain MN frequency data at 0 μM Hcy because cells cannot divide at this concentration in the absence of Met; and (ii) 50 μM Hcy may already be at the plateau of the dose–response curve. Future experiments should therefore study doses of <50 μM, where a dose-related effect may be observed which is also more consistent with the plasma concentrations of Hcy associated with moderate folate and vitamin B12 deficiency and elevated micronucleus frequency in vivo (Fenech et al., 1998).

Fasting plasma Hcy concentrations generally range from 7 to 25 μM (Brattström et al., 1988; Frostell et al., 1995) in apparently healthy subjects depending on folate, vitamin B12 and vitamin B6 status. High concentrations of L-DHcy caused a significant increase in the frequency of cells that were necrotic. Furthermore, Hcy concentrations positively correlated with the percentage of cells that were necrotic. This is in agreement with several reports of dose-dependent cytotoxicity to various cell types Hcy in vitro (Wall et al., 1980; Starkebaum and Harlan, 1986; Hultberg et al., 1997). A protective effect of catalase in these experiments suggests a role for H2O2 in the cellular damage caused by Hcy (Wall et al., 1980; Starkebaum and Harlan, 1986). Although H2O2 is an effective inducer of micronucleus formation (Crott and Fenech, 1999), it is clear that the major event in H2O2 exposure is necrosis (Crott and Fenech, 1999). The significantly higher necrosis in Met-treated cultures, which had much lower Hcy concentrations, does not support the hypothesis that Hcy is the cause of necrosis in lymphocyte cultures. In fact, the observed changes in necrosis may simply be a side effect of mitotic activity, with which necrosis was significantly and positively correlated ($r^2 = 0.11, P = 0.0005$).

Most of the pathologies that are associated with increased plasma Hcy concentrations involve the cardiovascular system. Recently it has been discovered that the tissue of the cardiovascular system (endothelial and smooth muscle) may have a limited ability to process Hcy due to a low expression of betaine:homocysteine methyl-transferase.) Since an important avenue of Hcy removal is absent in the cells of the cardiovascular system, these cells may be more susceptible to hyperhomocysteinemia than lymphocytes. For this reason, it seems necessary to investigate whether high concentrations of Hcy result in damage to the DNA in these cardiovascular cells and whether such DNA damage is one of the underlying causes of cardiovascular pathologies.

It must be noted that the great majority (99%) of Hcy in plasma occurs in its various oxidized forms, which are indistinguishable by most Hcy assays (Jacobsen, 1998). It is plausible, therefore, that the positive correlation observed between Hcy and micronucleus frequency in vivo (Fenech et al., 1997, 1998) is due to alternative potentially genotoxic forms of Hcy and not to Hcy itself. Experiments are currently under way to test this hypothesis.

In summary, this study shows that Hcy may induce a moderate increase in micronucleus formation in human lymphocytes in vitro. Further research is needed to verify these preliminary results and to determine the effects of chronic hyperhomocysteinemia, during B vitamin repletion, not only on lymphocytes, but on a diverse range of cell types, especially those of the human cardiovascular and nervous systems.

References


