Relationship between hepatic DNA damage and methylene chloride-induced hepatocarcinogenicity in B6C3F1 mice

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Methylene chloride (MC) induced DNA damage in freshly isolated hepatocytes from mice and rats, which was detectable as single-strand (ss) breaks by alkaline elution. The lowest in vitro concentration of MC needed to induce DNA damage in mouse hepatocytes (0.4 mM) was much lower than for rat hepatocytes (30 mM), and is close to the calculated steady-state concentration of MC in the mouse liver (1.6 mM) at a carcinogenic dose (4000 p.p.m. by inhalation). DNA ss breaks were also detectable in hepatocyte DNA from mice which had inhaled 4000 p.p.m. MC for 6 h, but not in hepatocyte DNA from rats similarly exposed. In studies with hepatocytes cultured overnight in the presence of buthionine sulfoximine to deplete glutathione (GSH), subsequent exposure to MC resulted in less DNA damage in the GSH-depleted cells. This shows that conjugation of MC with GSH is important in its activation to DNA-damaging species in the liver. The GSH pathway of MC metabolism produces two potential DNA-damaging species, formaldehyde and S-chloromethylglutathione (GSCH2Cl). Formaldehyde is known to cause DNA ss breaks in cells, however, the lowest concentration of formaldehyde required to induce a significant amount of DNA ss breaks in mouse hepatocytes (0.25 mM) is unlikely to be formed following in vitro or in vivo metabolism of MC at concentrations that induce similar amounts of DNA damage. That formaldehyde does not play a role in this DNA damage has been confirmed in experiments with CHO cells exposed to MC and an exogenous activation system from mouse liver (S9 fraction). Formaldehyde was responsible for the DNA-protein cross-linking effect of MC, but did not cause the DNA damage leading to ss breaks. These DNA ss breaks are likely to be caused by GSCH2Cl. The results suggest a genotoxic mechanism for MC carcinogenicity in the mouse liver, and support the proposal that the observed species differences in liver carcinogenicity result from differences in the amount of MC metabolism via the GSH pathway in the target organ.

Introduction

Methylene chloride (MC*, dichloromethane) is an ingredient in paint stripping and aerosol preparations and a solvent used in a wide variety of industrial applications. Inhalation of MC at 2000 and 4000 p.p.m. for 6 h per day over a 2 year period induces liver and lung tumours in B6C3F1 mice, but not in F344 rats or Syrian Golden hamsters (1). The reason for this carcinogenic effect is unknown. In in vitro assays MC is positive as a mutagen in bacteria (2,3) and yeast (4), but is not a mutagen in mammalian cells (5,6). The possible lack of metabolic activation of MC by the cells used in the latter assays has not been addressed. Unscheduled DNA synthesis (UDS) is not induced in rat hepatocytes after in vitro exposure (7). Some clastogenic effects have been reported in mammalian cells in vitro, but only at extremely high concentrations of MC which have little physiological relevance (8,9).

To date, conventional in vivo studies have been unable to conclusively provide a genotoxic explanation for MC-induced carcinogenicity in the mouse. UDS is not induced in the mouse liver after inhalation at doses used in the NTP study (10). Mice cytogenetic assays have been negative (11,12) except for a recent study showing marginal increases in chromosome damage, micronuclei and sister chromatid exchange (13). In addition, no significant effects have been observed in mouse liver which are commonly associated with non-genotoxic mechanisms of carcinogenesis, such as stimulation of mitogenesis or peroxisome proliferation (14,15). However, mitogenesis and cytotoxicity have been reported in the mouse lung (16,17). Although some authors have failed to observe base adducts in DNA isolated from mice treated with 14C-labelled MC (18,19), Casanova et al. (20) report the presence of DNA-protein cross-links in mouse liver. These probably arise from reaction of the MC metabolite formaldehyde with DNA, since they are found in DNA after formaldehyde exposure (21-23), though their relevance to MC carcinogenicity is unknown.

MC metabolism is well characterized. Cytochrome P450 metabolism occurs via oxidative dehalogenation producing formal chloride, which rapidly decomposes to give chloride ion and carbon monoxide (24,25). Conjugation of MC with glutathione (GSH) also occurs (26,27) yielding S-chloromethylglutathione (GSCH2Cl). Theta class glutathione-S-transferases (GST 5-5, 12-12) with a high affinity for MC have been purified from rat liver (28). GSCH2Cl has not been chemically isolated and breaks down rapidly to yield GSH and formaldehyde. The GSH pathway of MC metabolism is important in bacterial mutagenicity (29-32). Recently, the role of theta class GSTs in the activation of MC to DNA-damaging species has been confirmed by an increase in MC-induced mutations in Salmonella expressing rat liver GST 5-5 (33). GSH-mediated metabolism is also proposed to play a role in MC carcinogenicity in the mouse liver (34). The amount of MC metabolism via GSH conjugation in the mouse liver is relatively much greater than in non-target species, and is not saturable at high doses. By contrast, liver cytochrome P450 metabolism is saturated at doses well below those that induce tumours and has a lower capacity than the GSH pathway (35,36).

GSCH2Cl and formaldehyde produced by the GSH pathway of MC metabolism are both potentially DNA reactive species. The DNA-protein cross-linking detected in the mouse liver is due to formaldehyde (20), but in vivo effects that can be attributed to GSCH2Cl have not been described. A close analogue, the GSH conjugate of 1,2-dibromoethane (1,2-DBE; GSCH2CH2Br), is known to form adducts with DNA and to induce DNA single-strand (ss) breaks (37,38). An analogue of the MC-GSH

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conjugate, S-(1-acetoxyethyl)GSH, binds to 2'-deoxyguanosine in vitro (33). In this paper, we have investigated the role of the potentially DNA-damaging species GSCH₂Cl and formaldehyde in MC carcinogenicity using hepatocytes, in vitro studies and cultured CHO cells.

Materials and methods

MC (HPLC grade) was obtained from Fisons (Loughborough, UK), formaldehyde (Analar grade) from BDH (Poole, UK), and 1,2-D BE from Aldrich (Gillingham, UK). Proteinase K, collagenase and butyronitrocellulose (BSO) were obtained from Sigma (Poole, UK). Leibovitz L-15 medium and tryptophane phosphate broth were from ICN Biomedicals Ltd (High Wycombe, UK), and Ham’s F12 medium, Hank’s balanced salt solution (Ca/Mg-free) and fetal calf serum (heat inactivated) from Gibco (Paisley, UK). [methyl-³H]Thymidine (50 Ci/mmol) was obtained from Amersham (Aylesbury, UK). Male B6C3F1 mice (20–30 g, Charles River, Kent, UK) or male Alpk:APISD (AP) rats (200–250 g, Zeneca, Macclesfield, UK) were used. CHO-K1 cells were from Zeneca stock and liver S9 fraction was prepared from B6C3F1 mice using a standard procedure (39).

Rat hepatocytes were prepared by in situ cannulation of the hepatic portal vein and collagenase perfusion with recirculation (40). For mouse hepatocyte preparation, the perfusion buffer containing collagenase was passed only once through the liver. Rat hepatocytes were routinely obtained with a viability of >85% as determined by trypan blue exclusion. Mouse hepatocytes of similar viability were obtained by centrifugation of the isolated cells (initial viability 60–70%) through Percoll (41).

Hepatocytes prepared from a single liver (2 × 10⁶ per flask in 2 ml serum-free Leibovitz L-15 medium) were exposed in vitro to MC for 2 h at 37°C with gentle shaking in 25 ml Resciflasks (Pierce Scientific, Chester, UK). For rat hepatocytes, 5, 10 or 15 μl of MC was injected directly into the flask through the Teflon seal. For mouse hepatocytes 1–10 μl of a 1:10 dilution of MC in DMOSO was injected. The aqueous concentrations of MC were calculated from gas chromatographic measurements of MC in the head space of flasks incubated with medium but no cells. In vitro incubations were also performed with formaldehyde and 1,2-DBE. In vivo exposure to MC was by inhalation at 4000 p.p.m. or 4000 p.p.m. for 3 or 6 h, with the atmospheres monitored regularly by gas chromatographic analysis and compared to known standards (18).

Following exposure of the animals, hepatocytes were prepared as described.

DNA damage in hepatocytes was measured by alkaline elution through 25 mm diameter 2 μm pore size polycarbonate filters in a modified Swinnex filter unit (Millipore) as described by Kohn et al. (42). Cells (2 × 10⁶ per filter) were loaded, lysed, washed and eluted with tetrapropylammonium hydroxide-EDTA, pH > 12.1. Five fractions of 6 ml were collected at a flow rate of 0.3 ml/min. Filter DNA was recovered by heating the filters overnight at 60°C in elution buffer, and the DNA content of filters and fractions was measured by fluorimetric analysis using Hoechst 33258 (43).

Hepatocytes were cultured as described (40) in 25 cm² Falcon tissue culture flasks seeded with 2 × 10⁶ viable cells in 4 ml complete L-15 medium containing 10% fetal calf serum (CL-15). After incubation for 4 h at 37°C to allow cell attachment, the medium was replaced with 4 ml fresh CL-15 with or without 0.2 mM BSO. Following overnight incubation, the medium was replaced with CL-15 containing 5 mM MC (mouse cells) or 90 mM MC (rat cells) with or without 0.2 mM BSO, and the filters incubated, with tightly sealed caps, for a further 3 h. For measurement of DNA damage the cells were gently scrapped into 1 ml ice-cold PBS and loaded onto polycarbonate filters for alkaline elution analysis. Measurement of GSH content was performed using the assay of Hissin and Hilf (44) and total protein content measured using the technique of Bradford (45).

CHO-K1 cells were grown in Ham’s F12 medium containing 10% fetal calf serum in a humidified atmosphere containing 5% CO₂ and the DNA labelled by incubation with 0.25 μ Ci/ml [methyl-³H]Thymidine. Labelled cells (~ 2.5 × 10⁶) were exposed for 2 h to test compounds (50 mM MC, 0.125–0.5 mM formaldehyde or 1–10 mM 1,2-DBE) in tightly capped T-25 flasks containing 5 ml serum-free medium buffered to pH 7.4 with 20 mM HEPES. For incubations including mouse liver S9 fraction, the medium was supplemented with 1 mM glucose-6-phosphate, 1 mM NADP, 1 mM GSH and 2 mM CaCl₂. In some experiments concentrations of semicarbazide of up to 8 mM were included. After incubation, the cells were removed by gentle scraping into 1 ml Ca/Mg-free Hank’s balanced salt solution.

Half of the samples were lysed on polycarbonate filters and DNA damage analysed by alkaline elution as described, except that the amount of [³H]DNA remaining on the filters and in the eluate was determined directly by scintillation counting. The remaining samples of treated cells were lysed in 10 ml TCA (HCl, pH 7.4, 1% SDS at 65°C, and stored at -70°C. DNA-protein cross-linking in these cells was measured using the K-SDS precipitation method of Zhikhovitch and Costa (46).

Results

An increase in DNA ss breaks was observed in mouse and rat hepatocytes, measured by alkaline elution, following in vitro incubation with MC. In mouse hepatocytes the lowest concentration of MC needed to induce significant DNA ss breaks was 0.4 mM, compared to 30 mM for rat hepatocytes (Figure 1). These concentrations of MC were not cytotoxic to the cells as determined by trypan blue exclusion. A reduction in viability of 50% compared to control cells was observed at a concentration of 30 mM MC in mouse hepatocytes and 100 mM MC in rat hepatocytes. DNA damage induced by the MC metabolite formaldehyde was also measured in hepatocytes in vitro, with DNA ss breaks occurring at a concentration of 0.25 mM and above in mouse hepatocytes compared to 1 mM in rat hepatocytes (Figure 2). The result for rat hepatocytes is consistent with published work (47). Rat hepatocytes were very sensitive to DNA ss breaks induced by 1,2-DBE (Figure 3), with DNA damage detectable at 0.05 mM.

DNA damage in vivo was detectable in hepatocytes from mice exposed to an atmosphere of 4000 p.p.m. MC for 6 h, but not in hepatocytes from rats similarly exposed (Figure 4). DNA ss breaks were also found in hepatocytes from mice exposed to 4000 p.p.m. MC for 3 h, but not in mice exposed to 2000 p.p.m. MC for 6 h (results not shown). These exposures did not decrease the viability of the isolated hepatocytes compared to hepatocytes from control animals. Steady-state concentrations of MC in the liver during inhalation exposure were calculated using an established physiologically based pharmacokinetic model (PBPK) (48,49). This gave a liver concentration of 1.6 and 1.2 mM respectively for mice and rats exposed to 4000 p.p.m. MC.

The influence of GSH conjugation on the activation of MC.
Hepatic DNA damage and MC-induced carcinogenicity to DNA-damaging species was examined. When hepatocytes were cultured overnight in CL-15 medium with 0.2 mM BSO, the GSH content fell from 34.5 ± 0.7 to 14.5 ± 4.5 nmol GSH/mg protein in mouse hepatocytes and from 47.6 ± 0.9 to 14.7 ± 0.5 nmol GSH/mg protein in rat hepatocytes. These mean results from duplicate experiments are comparable to published values (47). One day old control hepatocytes were less sensitive to the DNA-damaging effect of MC, with the lowest concentration of MC needed to induce DNA ss breaks being 5 mM for mouse cells and 90 mM for rat cells. At these concentrations of MC there was a decrease in DNA ss breaks in the GSH-depleted hepatocytes (Figure 5).

No DNA ss breaks or DNA–protein cross-links were detected in CHO cells incubated with 60 mM MC for 2 h (Table I). However, by supplementing the incubation medium with B6C3F1 mouse liver S9 fraction, the S9-mediated metabolism of MC caused an increase in DNA ss breaks (Figure 6 and Table I) and DNA–protein cross-links (Table I). Formaldehyde and 1,2-DBE both induced DNA ss breaks in CHO cells in the absence of mouse liver S9 fraction, but DNA–protein cross-links were observed only with formaldehyde and not 1,2-DBE (Table I).

Addition of semicarbazide to the incubation medium inhibited formaldehyde-induced DNA ss breaks and DNA–protein cross-links in CHO cells (Table II). In the presence of S9 fraction, a higher concentration of formaldehyde was needed to induce both types of DNA damage (4 mM compared to 0.25 mM), and this damage was also inhibited by addition of semicarbazide. For MC metabolized exogenously by the S9 fraction, the DNA–protein cross-linking effect was completely abolished by addition of semicarbazide, whereas there was no effect on the DNA damage detectable as ss breaks.

Discussion
Several recent publications have extended the findings of the NTP review on MC carcinogenicity in B6C3F1 mice (reviewed 50).

Table I. DNA damage in CHO cells incubated with 1,2-DBE, formaldehyde (HCHO) and MC (+ S9 fraction from mouse liver)

<table>
<thead>
<tr>
<th></th>
<th>DNA–protein cross-links</th>
<th>DNA ss breaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (+ 1 ml S9)</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>0.125 mM HCHO</td>
<td>1.3 ± 0.1</td>
<td>22</td>
</tr>
<tr>
<td>0.25 mM HCHO</td>
<td>6.0 ± 0.8</td>
<td>77</td>
</tr>
<tr>
<td>0.5 mM HCHO</td>
<td>20.3 ± 1.3</td>
<td>80</td>
</tr>
<tr>
<td>60 mM MC</td>
<td>1.2 ± 0.2</td>
<td>2</td>
</tr>
<tr>
<td>60 mM MC (+ 0.25 ml S9)</td>
<td>3.6 ± 0.5</td>
<td>10</td>
</tr>
<tr>
<td>60 mM MC (+ 0.5 ml S9)</td>
<td>7.9 ± 2.0</td>
<td>26</td>
</tr>
<tr>
<td>60 mM MC (+ 1 ml S9)</td>
<td>13.0 ± 3.2</td>
<td>49</td>
</tr>
<tr>
<td>1 mM 1,2-DBE</td>
<td>1.1 ± 0.1</td>
<td>20</td>
</tr>
<tr>
<td>5 mM 1,2-DBE</td>
<td>1.3 ± 0.2</td>
<td>60</td>
</tr>
<tr>
<td>10 mM 1,2-DBE</td>
<td>1.2 ± 0.1</td>
<td>89</td>
</tr>
</tbody>
</table>

*[^3]H]DNA in K-SDS precipitate (treated cells)/(control cells). Results are ± SD from triplicate samples in a single experiment (mean value for control precipitates ~ 450 c.p.m.).

% total[^3]H]DNA eluted from filter for treated cells relative to control cells. Alkaline elution was performed as in Figure 6. Results are means from duplicate filter and fraction measurements in a single experiment.
Table II. Effect of semicarbazide (SC) on HCHO and MC-induced DNA damage in CHO cells (± S9 fraction from mouse liver)

<table>
<thead>
<tr>
<th>Condition</th>
<th>DNA–protein cross-links $a,b$</th>
<th>DNA ss breaks $a,b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (+ 1 ml S9, 5 mM SC)</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>0.5 mM HCHO</td>
<td>20.3 ± 1.3</td>
<td>80</td>
</tr>
<tr>
<td>0.5 mM HCHO (+ 0.5 mM SC)</td>
<td>2.6 ± 0.2</td>
<td>26</td>
</tr>
<tr>
<td>2 mM HCHO (+ 1 ml S9)</td>
<td>1.9 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>4 mM HCHO (+ 1 ml S9)</td>
<td>15.7 ± 0.6</td>
<td>35</td>
</tr>
<tr>
<td>4 mM HCHO (+ 1 ml S9, 8 mM SC)</td>
<td>0.9 ± 0.1</td>
<td>5</td>
</tr>
<tr>
<td>60 mM MC</td>
<td>1.2 ± 0.9</td>
<td>0</td>
</tr>
<tr>
<td>60 mM MC (+ 1 ml S9)</td>
<td>13.5 ± 2.9</td>
<td>42</td>
</tr>
<tr>
<td>60 mM MC (+ 1 ml S9, 5 mM SC)</td>
<td>1.6 ± 0.2</td>
<td>38</td>
</tr>
</tbody>
</table>

$a,b$See legend to Table I.

A new in vivo study has confirmed liver and lung tumours (51) without cytotoxicity or stimulation of cell proliferation (15). Whilst the results in the liver are consistent with earlier work (14), the absence of cytotoxicity and increased cell division in the lungs of mice exposed to MC (52) is in contrast to the observations of Foster et al. (16,17). Using DNA samples derived from the liver and lungs, Devereux et al. (53) demonstrated that H-ras and K-ras codon 61 mutations, both in frequency and in base changes. As a result of this study the authors suggested that MC-induced liver tumours were induced by an unknown promotional mechanism. Because of the limited number of tumour samples available, no conclusions were drawn about the mechanism in the lung. Therefore, in spite of these recent efforts, the mechanism of action of MC remains elusive.

The results presented in this paper show that MC is genotoxic in hepatocytes. The lowest concentration of MC required to induce DNA damage in freshly isolated mouse hepatocytes in vitro (0.4 mM) was significantly lower than for rat hepatocytes (30 mM). These differences in sensitivity observed in vitro were reflected in the in vivo results, with DNA ss breaks detectable in hepatocytes from mice exposed to 4000 p.p.m. MC by inhalation for 3 or 6 h but not in hepatocytes from rats similarly exposed. This DNA damage induced in vitro and in vivo was not related to cytotoxicity, which is consistent with a lack of cytotoxicity in published bioassays.

Using an established PB-PK model for MC in rodents (48,49), the calculated steady-state concentration of MC in the livers of mice exposed to 4000 p.p.m. MC by inhalation (1.6 mM) is close to the concentration needed to induce DNA ss breaks in vitro (0.4 mM). By contrast, the lowest concentration of MC needed to induce DNA ss breaks in rat hepatocytes in vitro (30 mM) corresponds to a calculated in vivo exposure of 86 000 p.p.m., which is several times the lethal dose (LD$_{50}$ = 15 000 p.p.m.). Consequently DNA ss breaks (and tumours) are not seen in the rat in vivo. Therefore, these results suggest a genotoxic mechanism for MC carcinogenicity in the mouse, and offer an explanation for the observed species differences.

In support of this proposal, the DNA-damaging effect of MC in hepatocytes in vitro was found to depend upon metabolism via the GSH pathway. Fewer MC-induced DNA ss breaks occurred in hepatocytes preincubated with BSO to deplete GSH. This result is consistent with the observation that MC is metabolized to a bacterial mutagen via GSH metabolism (29–33). In rodents a number of studies have linked the GSH pathway of MC metabolism to the induction of liver and lung tumours in mice, but not in rats or hamsters (reviewed 34). The decrease in sensitivity to MC-induced DNA damage in the control cultured hepatocytes compared to freshly isolated cells may reflect loss of metabolic activity during culture.

The results offer further insight into the nature of the DNA-damaging species. The two reactive electrophiles generated by GSH metabolism of MC are formaldehyde and the conjugate with GSH (GSCH$_2$Cl). The in vitro concentration of formaldehyde needed to induce DNA ss breaks in hepatocytes (0.25 mM in the mouse and 1 mM in the rat) is unlikely to be reached in the cell following metabolism of MC. Also, this species difference is insufficient to account for the differences in sensitivity observed for MC-induced DNA damage.

In addition to DNA ss breaks, formaldehyde induces DNA–protein cross-links. Both types of damage were induced in DNA from CHO cells incubated with formaldehyde, and with MC metabolized exogenously by mouse liver S9. There was no DNA damage in CHO cells incubated with MC alone, suggesting a lack of metabolic activation and providing an explanation for the absence of MC-induced mutations in these cells (5,6). In contrast 1,2-DBE, which is metabolized to a DNA-binding GSH conjugate (38), is mutagenic (54) and causes DNA ss breaks (Table I) in CHO cells, without requirement for S9 activation. Conjugation of 1,2-DBE with GSH is catalysed by different GSTs (primarily alpha class 2-2, ref. 55) compared to MC (theta class 5-5, 12-12, refs 28,33), and the results suggest that CHO cells are competent in this metabolism.

Addition of semicarbazide to the incubation medium inhibited formaldehyde-induced DNA ss breaks and DNA–protein cross-links, but inhibited only the DNA–protein cross-links induced by MC. Therefore, formaldehyde was responsible for the DNA–protein cross-linking effect of MC but not the major part of the damage leading to DNA ss breaks. DNA–protein cross-links have not been assayed in hepatocyte DNA in this study, but have been observed in the mouse liver following exposure to MC (20).

The products of GSH conjugation with 1,2-DBE and 1,2-DCE, GSCH$_2$CH$_2$Br(Cl), form DNA adducts with both adenine and guanine (55,57). These compounds are also potent inducers of DNA ss breaks both in vivo (37) and in vitro (Figure 3). At present there is no direct evidence for the formation of an adduct between the GSH conjugate of MC and DNA in vivo, but the inherent chemical reactivity of GSCH$_2$Cl and the structural analogy to the conjugates of the 1,2-dihaloalkanes suggest that GSCH$_2$Cl may be responsible for the DNA ss breaks seen after exposure to MC. Thus it appears that GSH-mediated metabolism of MC causes DNA ss breaks via GSCH$_2$Cl and DNA–protein cross-links via formaldehyde. This combination may provide an explanation for the lack of effect of MC in the mouse liver UDS assay (10). A number of studies have shown that formaldehyde inhibits DNA repair processes (reviewed 58). Inhibition of DNA repair may explain why formaldehyde can potentiate the mutagenicity of physical and chemical carcinogens (59).

In conclusion, DNA damage has been observed in mouse hepatocytes in vitro and in vivo, but not in the rat hepatocytes in vivo. The nature of this damage, the correlation with GSH-mediated metabolism of MC and the species dependence suggest that it plays a fundamental role in the mechanism of action of MC as a mouse liver carcinogen. Further studies are in progress to investigate the possible inhibitory effect of formaldehyde on the repair of MC-induced DNA damage, and to determine if similar effects occur in the mouse lung.
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References


metabolism of dihalomethanes to carbon
monoxide, n.


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