The distribution of Theta-class glutathione S-transferases in the liver and lung of mouse, rat and human

Guy W. MAINWARING, Sally M. WILLIAMS, John R. FOSTER, Jonathan TUGWOOD and Trevor GREEN*

Zeneca Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, U.K.

Two murine Theta-class glutathione S-transferases (GSTs), mGSTT1 and mGSTT2, have been cloned and sequenced. The murine cDNAs, together with the published sequences of the rat and human enzymes, were used to design oligonucleotide probes in order to determine the distribution of mRNA for these enzymes in the liver and lung of rat, mouse and human. The mRNA distribution was compared with that of enzyme protein determined with an antibody to rat GSTT2–2. Both the antibody and the oligonucleotide probes gave the same distribution patterns. Both enzymes were present at significantly higher concentrations in mouse tissues than in rat or human tissues. In mouse liver, both enzymes were localized in specific cell types and in nuclei. Although the distribution of GSTT2–2 in rat liver was similar to that seen in the mouse, GSTT1–1 was not localized in a specific cell type or in the nuclei of either rat or human liver. In the lungs, very high concentrations of the Theta enzymes were present in mouse-lung Clara cells and ciliated cells, with much lower levels in the Clara cells only of rat lung. Low levels of human transferase GSTT1–1 were detected in a small number of Clara cells and ciliated cells at the alveolar/bronchiolar junction. The relative activities between species, and the cellular and sub-cellular distribution within the liver and lungs of each species, provides an explanation for the species-specificity of methylene chloride, a mouse-specific carcinogen activated by glutathione S-transferase GSTT1–1.

INTRODUCTION

The soluble glutathione transferases (GST; EC 2.5.1.18) are a supergene family of dimeric proteins which conjugate glutathione to a wide range of substrates [1,2]. In mammals, there are currently five known families of soluble GSTs, Alpha, Mu, Pi, Sigma and Theta, each with distinct catalytic and structural properties [3]. The Theta family has been identified relatively recently in rat, mouse and human [4–7], and at present three rat Theta enzymes are known, rGSTT1–1, rGSTT2–2 and rGSTT3–3 (previously GSTs 5–5, 12–12 and 13–13), two mouse enzymes, mGSTT1–1 and mGSTT2–2, and two human enzymes, hGSTT1–1 and hGSTT2–2 [3]. Sequencing has revealed that the GSTT1–1 enzymes from mouse, rat and human are orthologous proteins, as are the GSTT2–2 proteins. Relatively little is known about the distribution of this family across species or within the tissues of any individual species.

The GSTs have been implicated in the metabolic activation of a range of xenobiotics, both in the liver and the lung, and in other tissues such as the brain and kidney [8]. Some of these activation reactions have resulted in the development of tumours in laboratory animals. For example, methylene chloride is known to cause cancer in the lungs and livers of mice by a mechanism that involves a glutathione metabolite formed by the mouse Theta enzyme GSTT1–1 [7,9,10]. Although enzymes equivalent to this mouse enzyme are known to exist in the rat (rGSTT1–1) and in humans (hGSTT1–1), increases in lung and liver cancer are not seen in rats (or hamsters) exposed to methylene chloride [11], nor is there any evidence for an increase in cancer in exposed human populations, suggesting that either the activity or distribution of these enzymes differs markedly between species. Assessment of enzyme activity based on whole tissue homogenates suggests that the former is true, the enzyme activity in mouse tissues being an order of magnitude greater than that found in rat, hamster or human tissues with methylene chloride as the substrate. Assays based on whole tissue homogenates do, however, have a fundamental weakness in that they fail to identify specific cells within organs with heterogeneous cell populations that may contain high enzyme activity. Identifying cells with high activity plays a key part in accurately predicting human risk when using animal data during the risk assessment process.

Thus, although biochemical and enzymological evidence exists for the presence of these enzymes in the tissues of rodents and humans, their distribution has not been plotted either in rodent or human tissues due to a lack of suitable molecular probes and antibodies for this class of GST enzymes. The nucleotide sequence for the major types of the Theta-class GST enzymes have recently become available, and oligonucleotide probes have been manufactured against the mRNA of the GSTT1–1 and GSTT2–2 enzymes from mouse and rat, and the GSTT1–1 enzyme from humans. These probes have been used in the current study to plot the distribution of the mRNAs in the liver and lung of mouse, rat and human. In addition, an antibody which was raised against recombinant rat Theta enzyme rGSTT2–2 was available to the authors and has been used in the current investigation to immunolocalize the GSTT2–2 enzymes in the same tissues as those used in the mRNA study.

MATERIALS AND METHODS

Source of liver and lung

Samples of liver and lung were taken from male B6C3F1 mice and male F344 rats. Liver slices, 2–3 mm thick, were fixed in 4% paraformaldehyde for 24 h before being dehydrated and embedded in paraffin wax. The samples of lung were fixed by

Abbreviations used: GST, glutathione S-transferases; FITC, fluorescein isothiocyanate.

The nucleotide sequence data reported in this paper for mouse Theta GSTT1 and GSTT2 appear in the EMBL and GenBank Databases under accession numbers X98055 and X98056 respectively.

* To whom correspondence should be addressed.

inflation with 4 % paraformaldehyde followed by further fixation for 24 h before being cut into 2–3 mm thick slices, dehydrated and embedded in paraffin wax.

Whole human lung tissue ($n = 4$) was obtained from the International Institute for the Advancement of Medicine (Exton, PA, U.S.A.) and lung sections ($n = 7$) from Dr T. Tetley (Department of Medicine, Charing Cross Hospital, London, U.K.). Human liver samples ($n = 5$) were obtained from the Liver Unit at Queen Elizabeth Hospital, Birmingham, U.K. Samples of the latter were cut into 2–3 mm slices and fixed in 4 % paraformaldehyde for 24 h before being dehydrated and embedded in paraffin wax.

Immunocytochemistry

All tissue sections were immunostained within 24 h of cutting to avoid variability due to storage. All the sections were cut and floated out onto a water bath at 40 °C. This was deliberately cooler than that used for routine wax sectioning to avoid heat denaturation of the antigenic epitopes. The sections were also dried at room temperature overnight for the same reason.

An antibody raised in rabbits against a recombinant rat GSTT2–2 was obtained from Dr. John Hayes (Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, Scotland, U.K.). The antibody gave a single band of the correct molecular mass on Western blots of mouse and rat liver cytosol. All sections were incubated in a 1:100 dilution of the antibody overnight at 4 °C, and a horseradish peroxidase-conjugated avidin methodology followed by diaminobenzidine tetrahydrochloride was applied to detect the sites of antibody binding. Controls consisted of sections incubated as per the test sections with the omission of the primary antibody. Endogenous peroxidase activity in both the lung and liver was destroyed by pre-incubation with hydrogen peroxide in methanol (100 μl of 30 % hydrogen peroxide in 50 ml of methanol). All sections were counterstained in haematoxylin, dehydrated and embedded in DPX mountant (BDH Chemicals).

Preparation of Theta mGSTT1 and mGSTT2 cDNA

Total RNA was isolated from $B_6C_3F_1$ mouse liver using standard protocols [12], and poly(A)$^+$ RNA was prepared by oligo(dT) affinity chromatography (Pharmacia LKB, Uppsala, Sweden). Oligonucleotide discovery primers were based on the primary amino acid sequences obtained from purified mGSTT1–1 and mGSTT2–2 [7] and on the published nucleotide sequences obtained from rat [13,14] (mGSTT1: sense strand ATGGTGCTGGAGCTCTATCTGGAT, antisense strand TCACTGGATCATGGTCAGCACTCT; and mGSTT2: sense strand ATGGGTTTGGAGCTCTACCTGGAC, antisense strand TCAGGGAATCCTGGCAATTCGAAG). These primers were used to amplify the coding region of the mouse GSTT1 and GSTT2 from mouse liver cDNA prepared by reverse transcription of the poly(A)$^+$ RNA using reverse transcriptase (Amersham International, Little Chalfont, Bucks., UK). First-strand cDNA was subjected to five cycles (94 °C for 1 min, 46 °C for 2 min, 72 °C for 3 min) of initial amplification followed by 34 cycles (94 °C for 1 min, 46 °C for 2 min, 72 °C for 3 min, and finally 72 °C for 6 min) in a 50 μl reaction mixture containing 50 pmol of each primer, 200 μM dNTPs, 2 units of *Taq* polymerase and 1 × *Taq* polymerase buffer (Promega, Southampton, U.K.).

The resulting PCR products, approx. 740 bp in length, were ligated into the PCR11 vectors (Invitrogen, De Schelp, The Netherlands) and competent *Escherichia coli* INVαF′ were transformed and grown overnight at 37 °C. The cDNA inserts were excised from the PCR11 vector by *Eco*RI digestion and both strands of the coding region of the Theta GSTs were sequenced by the dideoxy termination method [15].

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1
 M  V  L  E  L  Y  L  D  L  L  S  Q  P  C  R  A  I  Y  I  F
ATG GTG CTG GAG CTC TAT CTG GAT CTG CTG TCG CAG CCC TGT CGC GCC ATT TAT ATC TTC
21
 A  K  K  N  N  I  P  F  Q  M  H  T  V  E  L  R  K  G  E  H
GCC AAG AAG AAC AAT ATC CCG TTC CAG ATG CAC ACG GTG GAG CTG CGC AAG GGT GAG CAC
41
 L  S  D  A  F  A  R  V  N  P  M  K  K  V  P  A  M  M  D  G
CTC AGC GAT GCG TTT GCC CGG GTG AAC CCC ATG AAG AAG GTA CCA GCC ATG ATG GAT GGT
61
 G  F  T  L  C  E  S  V  A  I  L  L  Y  L  A  H  K  Y  K  V
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81
 P  D  H  W  Y  P  Q  D  L  Q  A  R  A  R  V  D  R  Y  L  A
CCT GAC CAC TGG TAC CCC CAA GAC CTG CAG GCT CGT GCT CGT GTA GAC GAG TAC CTG GCA
101
 W  Q  H  T  G  L  R  R  S  C  L  R  A  L  W  H  K  V  M  F
TGG CAG CAT ACG GGC CTT CGG AGA AGC TGC CTC AGG GCC CTG TGG CAT AAG GTG ATG TTC
121
 P  V  F  L  G  E  Q  I  P  P  E  T  L  A  A  T  L  A  E  L
CCT GTT TTC CTT GGT GAG CAA ATA CCT CCT GAA ACA CTG GCA GCC ACG TTG GCA GAA CTG
141
 D  V  N  L  Q  V  L  E  D  K  F  L  Q  D  K  D  F  L  V  G
GAT GTT AAC CTA CAG GTG CTT GAA GAC AAG TTC CTC CAG GAC AAA GAC TTC CTT GTT GGG
161
 P  H  I  S  L  A  D  L  V  A  I  T  E  L  M  H  P  V  G  G
CCC CAC ATC TCC CTG GCC GAC TTG GTG GCC ATC ACA GAG CTG ATG CAT CCT GTA GGT GGT
181
 G  C  P  V  F  E  G  H  P  R  L  A  A  W  Y  Q  R  V  E  A
GGC TGC CCA GTC TTT GAA GGG CAT CCC AGG CTG GCT GCA TGG TAC CAG CGA GTG GAG GCA
201
 A  V  G  K  D  L  F  R  E  A  H  E  V  I  L  K  V  K  D  C
GCT GTG GGG AAG GAC CTC TTC CGG GAA GCC CAT GAA GTC ATC CTG AAG GTG AAG GAC TGT
221
 P  P  A  D  L  I  I  K  Q  K  L  M  P  R  V  L  T  M  I  Q
CCC CCT GCT GAC CTC ATC ATA AAG CAG AAG CTG ATG CCC AGA GTG CTG ACC ATG ATC CAG

TGA
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Figure 1 Cloning of mouse Theta GSTT1 from mouse liver

Numbers refer to the amino acid residues (single letter code) which are deduced from the nucleotide sequence of mouse Theta GSTT1 typed immediately below. Nucleotide sequences underlined are the regions to which the mRNA probes were prepared.

In situ hybridization

Oligonucleotide anti-sense probes (30–50mer) complementary to three differing regions of the protein nucleotide sequences of rat GST1–1 and 2–2 [13,14], mouse GSTT1–1 and 2–2 (Figures 1 and 2), and human GSTT1–1 [16] (nucleotides 0–34, 350–379, 567–723) were synthesized and labelled with fluorescein diisothiocyanate at the 5′ end (Fluoroprime, Pharmacia LKB, Uppsala, Sweden).

As with the immunocytochemistry protocol, all tissue sections were stained within 24 h of *in situ* hybridization to avoid variability due to section storage. All the sections were also cut and floated out onto a water bath at 40 °C and subsequently dried at room temperature overnight. All reagents, buffers and glassware were, where possible, autoclaved to kill possible exogenous RNAase activity, and gloves were worn at all times during the procedure to avoid contaminating the sections and apparatus and denaturing the mRNA present in the sections. Diethyl pyrocarbonate was also added to all solutions to inhibit RNAase activity. After de-waxing, all sections were treated with 0.2 M HCl for 30 min, followed by proteinase K at concentrations from 2 to 20 mg/ml. It was found necessary always to use a range of concentrations of proteinase K to compensate for differences in the degree of fixation experienced by the tissues. This step was found to have the most influence on the successful outcome of the *in situ* hybridization protocol. The sections were post-fixed in 0.4 % paraformaldehyde for 20 min and then incubated for 1 h at 37 °C in a pre-hybridization solution [0.08 ml of 0.1 % diethyl pyrocarbonate in water, 0.12 ml of 5 M sodium chloride, 0.20 ml of 50 % (w/v) dextran sulphate, 0.50 ml of formamide and 0.10 ml of PE buffer, which consisted of 0.5 M Tris/HCl containing 1 % (w/v) sodium pyrophosphate, 2 % (w/v) polyvinyl-

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1
 M  G  L  E  L  Y  L  D  L  L  A  Q  P  S  R  A  V  Y  I  F
ATG GGT TTG GAG CTC TAC CTG GAC CTG CTG TCA CAA CCC AGC CGC GCT GTC TAC ATC TTC
21
 A  K  K  N  G  I  P  F  Q  T  R  T  V  D  I  L  K  G  Q  H
GCC AAG AAG AAT GGC ATC CCC TTC CAG ACG CGT ACC GTG GAT ATA CTC AAA GGG CAG CAC
41
 M  S  E  Q  F  S  Q  V  N  C  L  N  K  V  P  V  L  K  D  G
ATG AGC GAG CAA TTC TCC CAG GTG AAC TGC TTA AAC AAA GTT CCT GTA CTC AAA GAC GGA
61
 S  F  V  L  T  E  S  T  A  I  L  I  Y  L  S  S  K  Y  Q  V
AGC TTC GTG TTG ACC GAA AGC ACA GCC ATC TTG ATT TAC CTG AGT TCC AAG TAC CAG GTG
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 A  D  H  W  Y  P  A  D  L  Q  A  R  A  Q  V  H  E  Y  L  G
GCA GAC CAC TGG TAC CCG GCC GAC CTA CAG GCC CGT GCC CAA GTC CAC GAA TAC CTG GGC
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 W  H  A  D  N  I  R  G  T  F  G  V  L  L  W  T  K  V  L  G
TGG CAT GCC GAC AAC ATC CGT GGT ACT TTC GGA GTG CTC CTA TGG ACC AAG GTG TTG GGG
121
 P  L  I  G  V  Q  V  P  Q  E  K  V  E  R  N  R  D  R  M  V
CCA CTC ATT GGG GTC CAG GTT CCC CAG GAG AAG GTG GAA CGG AAC AGA GAT AGA ATG GTC
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 L  V  L  Q  Q  L  E  D  K  F  L  R  D  R  A  F  L  V  G  Q
CTG GTT CTG CAA CAG CTG GAG GAC AAG TTC CTC AGG GAC AGG GCC TTC CTT GTT GGC CAG
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 Q  V  T  L  A  D  L  M  S  L  E  E  L  M  Q  P  V  A  L  G
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181
 Y  N  L  F  E  G  R  P  Q  L  T  A  W  R  E  R  V  E  A  F
TAT AAC CTG TTT GAG GGA CGG CCT CAG CTG ACA GCA TGG CGA GAG AGG GTG GAG GCG TTC
201
 L  G  A  E  L  C  Q  E  A  H  S  T  I  L  S  I  L  G  Q  A
TTG GGT GCT GAG CTG TGT CAG GAG GCT CAT AGC ACC ATC CTG AGC ATC CTG GGA CAG GCA
221
 A  K  K  M  L  P  V  P  P  P  E  V  H  A  S  M  Q  L  R  I
GCC AAG AAA ATG TTA CCA GTA CCC CCT CCG GAG GTC CAT GCC AGC ATG CAG CTT CGA ATT
241
 A  R  I  P
GCC AGG ATT CCC TGA
```

Figure 2 Cloning of mouse Theta GSTT2 from mouse liver

Numbers refer to the amino acid residues (single letter code) which are deduced from the nucleotide sequence of mouse Theta GSTT2 typed immediately below. Nucleotide sequences underlined are the regions to which the mRNA probes were prepared.

pyrrolidone, 2 % (w/v) Ficoll and 50 mM EDTA] in a humidity chamber. After removal of this solution from the sections, they were incubated overnight in hybridization solution (0.06 ml of 5 M sodium chloride/0.05 ml of PE buffer/0.10 ml of dextran sulphate/0.25 ml of formamide) to which had been added 0.5 μg/ml of each of the fluorescein isothiocyanate (FITC)-labelled anti-sense oligonucleotide probes. Following removal of the hybridization mixture, the sections were incubated in a 1 in 50 dilution of an alkaline phosphatase-conjugated anti-FITC antibody (Boehringer-Mannheim, Mannheim, Germany) for a further one hour at room temperature. The sites of binding of the anti-sense probe were localized using 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium as a substrate for the alkaline phosphatase enzyme conjugated to the anti-FITC antibody used to locate the bound oligonucleotides. All sections were dehydrated and embedded in DPX.

Metabolism of methylene chloride by human lung tissue

The ability of human lung fractions to metabolize methylene chloride was measured by assaying for formaldehyde production, which is specific for metabolism of this chemical by the Theta GSTs [17]. A 25 % (w/v) homogenate of lung tissue was prepared in 0.25 M sucrose/20 mM Tris/HCl/5.4 mM EDTA buffer, pH 7.4, using a Polytron homogenizer. The homogenate was centrifuged at 100000 ***g*** for 60 min. An aliquot of the supernatant was taken for protein analysis [18], and 0.20 ml was used for assay with methylene chloride (3 μl) in 1.3 ml of 200 mM Tris/HCl buffer, pH 7.4, containing 5 mM GSH. After 10 and 20 min incubation at 37 °C in sealed vials, aliquots (1.0 ml) were taken and protein was precipitated with 20 % trichloroacetic acid (0.1 ml). The supernatant was assayed for formaldehyde using the procedure of Nash [19].

RESULTS

Immunolocalization of GSTT2–2

Protein reactive to Theta rGSTT2–2 antibody was localized in liver and lung sections from mice, rats and humans. In male B6C3F1 mice the highest concentrations were found in the limiting plate hepatocytes of the central vein in the liver. There was also strong staining present in the central vein hepatocytes in general and in the nuclei of some but not all cells. The periportal hepatocytes remained largely unstained (Figure 3). In contrast to the low levels of staining observed in the periportal hepatocytes, the adjacent interlobular bile-duct epithelial cells showed immunostaining at levels comparable to that seen in the central vein hepatocytes.

Although the distribution of GSTT2–2 protein in the male F344 rat liver was comparable to that in the mouse, with the highest concentrations in the limiting plate hepatocytes around the central vein and in the nuclei of some cells, the overall amounts in the rat liver were considerably less than those observed in the mouse. The levels in the interlobular bile ducts were similar to those seen in the rest of the rat liver (Figure 3). Protein reactive to the rGSTT2–2 antibody in human liver was expressed in very low levels and was evenly distributed across the liver lobule, quite unlike the distribution seen in either the mouse or the rat. There was no evidence of nuclear staining, but the interlobular bile-duct epithelium showed levels of immunostaining comparable to that seen in the mouse limiting plate hepatocytes and bile-duct epithelium (Figure 3).

In the mouse lung, mGSTT2-2 was localized in the bronchiolar epithelium and in the type II alveolar cells of the lung parenchyma. Within the bronchiolar epithelium itself, the apical cytoplasm of Clara cells contained the highest concentrations of reaction product, with significant amounts also present in the ciliated bronchiolar cells (Figure 3). Rat lung contained much less GSTT2–2 than the mouse, and the protein that was present was mainly in the Clara cells of the bronchiolar epithelium while ciliated cells showed little, if any, GSTT2–2 (Figure 3). The type II alveolar cells also had low levels of GSTT2–2.

Human lung immunostained with the rGSTT2–2 antibody showed the protein to be present in the ciliated bronchiolar cells, alveolar macrophages and alveolar Type II cells at low concentrations, which were comparable to those seen in the rat (Figure 3).

Cloning of two Theta GSTs from the $B_6C_3F_1$ mouse

The two mouse liver Theta GST clones (Figures 1 and 2) had greater than 90 % homology to the corresponding rat sequences, rGSTT1 and rGSTT2, and had approx. 60 % sequence homology towards each other. The murine Alpha-, Mu- and Pi-class GSTs show close sequence homology to the equivalent GST families found in rat [20–22] and share at least 20 % identity between families, whereas the sequence identity to the Theta family is only about 7 % [13]. In fact, rGSTT1 was found to have greater similarity to a dehalogenase enzyme obtained from *Methylobacterium* sp. DM4, an enzyme which is also capable of metabolizing methylene chloride [7,13,23].

In situ hybridization

The oligonucleotide anti-sense probes raised to three differing regions of the protein nucleotide sequences of rat GSTT1–1 and GSTT2–2, mouse GSTT1–1 and GSTT2–2, and human GSTT1–1 were used to study the distribution of the mRNAs for these enzymes. The individual probes raised to the three regions for each enzyme gave identical distribution patterns, and the

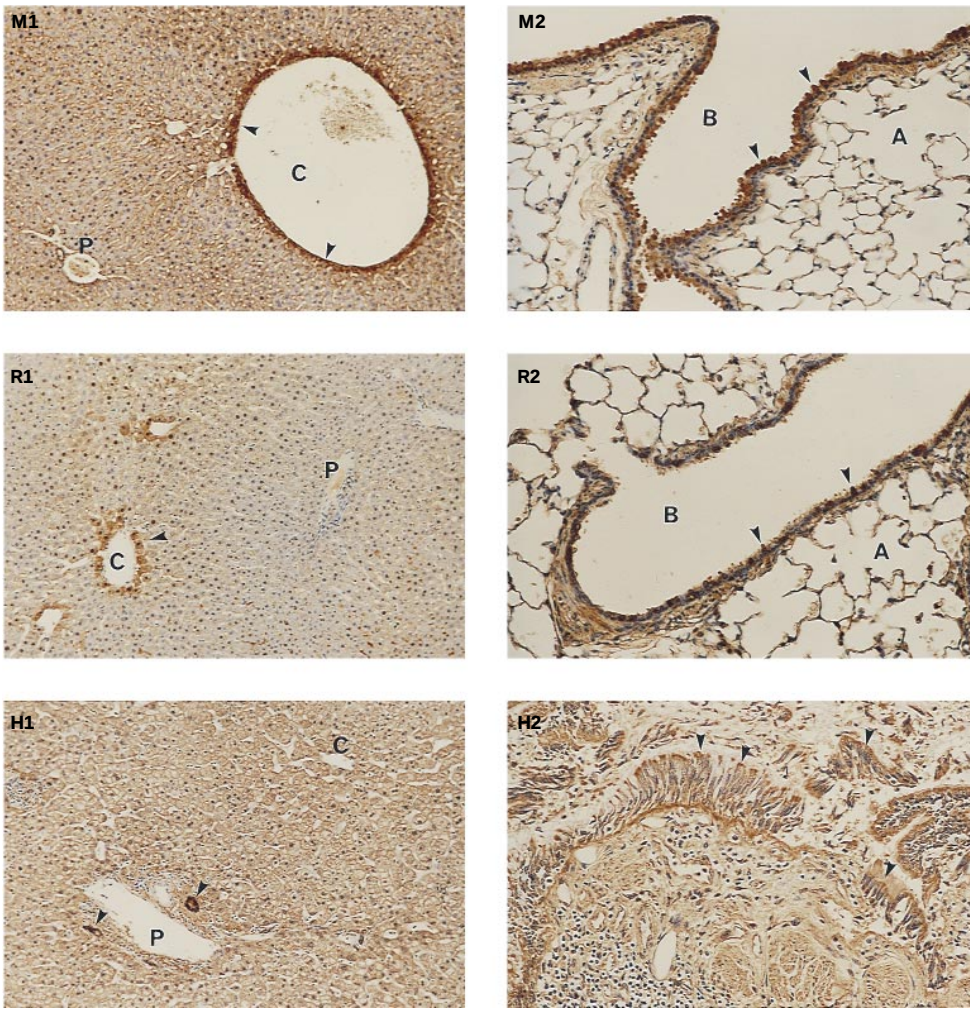

Figure 3 Immunolocalization of GSTT2–2 in mouse liver (M1) and lung (M2), in rat liver (R1) and lung (R2), and in human liver (H1) and lung (H2)

In the lung sections: A corresponds to the alveolus; and B, the bronchiolar lumen. Arrows mark Clara cells in rat and mouse lung sections and the bronchiolar epithelium in the human lung section. In the liver sections: C corresponds to the central vein and P the portal vein. Arrows mark the limiting plate hepatocytes in the rat and mouse sections and the bile ducts in the human section.

data shown were obtained with a combination of all three probes.

The distribution of mRNA to both GSTT1–1 and GST2–2 was virtually identical in the tissues examined, and consequently only data for GSTT1–1 are shown (Figure 4). Within the liver of the mouse, mRNA was localized to the limiting plate hepatocytes around the larger central veins, in nuclei, and in lesser amounts in the centrilobular cells in general (Figure 4). Although the periportal hepatocytes were generally negative for the mRNAs of either enzyme, the bile-duct epithelial cells showed high concentrations of the mRNA for both enzymes. In rat liver, mRNA to both Theta-class GSTs was present, but at considerably lower levels than in the mouse. The pattern of distribution was similar to the mouse in that the concentration of mRNA was highest in the central vein hepatocytes and bile-duct epithelial cells (Figure 4). However, there was no enhanced staining in the limiting plate hepatocytes or in nuclei as seen in the mouse liver. In the human liver samples the concentration of the mRNAs for both enzymes was very low, with an even distribution throughout the liver lobule (Figure 4). No major differences were noted in the levels of mRNA between individual liver samples.

In the lung of the male B6C3F1 mouse, the mRNAs for both GSTT1–1 and GSTT2–2 were present at very high concentrations in the bronchiolar epithelium, with lesser amounts being present in the type II cells (Figure 4). In contrast to the situation seen using the antibodies to the GSTT2–2 form, *in situ* hybridization showed that the mRNA was present at highest concentrations in the ciliated cells, with lesser, although still high, concentrations being present in the Clara cells. It is estimated that the amounts present in the individual cells of the bronchiolar epithelium were higher than those present in the individual hepatocytes of the liver, although the numbers of cells containing the mRNAs would be considerably less in the mouse lung than in the liver.

In the lungs of rats, the mRNAs for both Theta-class enzymes were present in very low concentrations and were limited to Clara cells (Figure 4). Of the human lung samples examined, hGSTT1–1 mRNA was only detectable in one of those samples. Low levels were present in Clara cells and ciliated cells at the

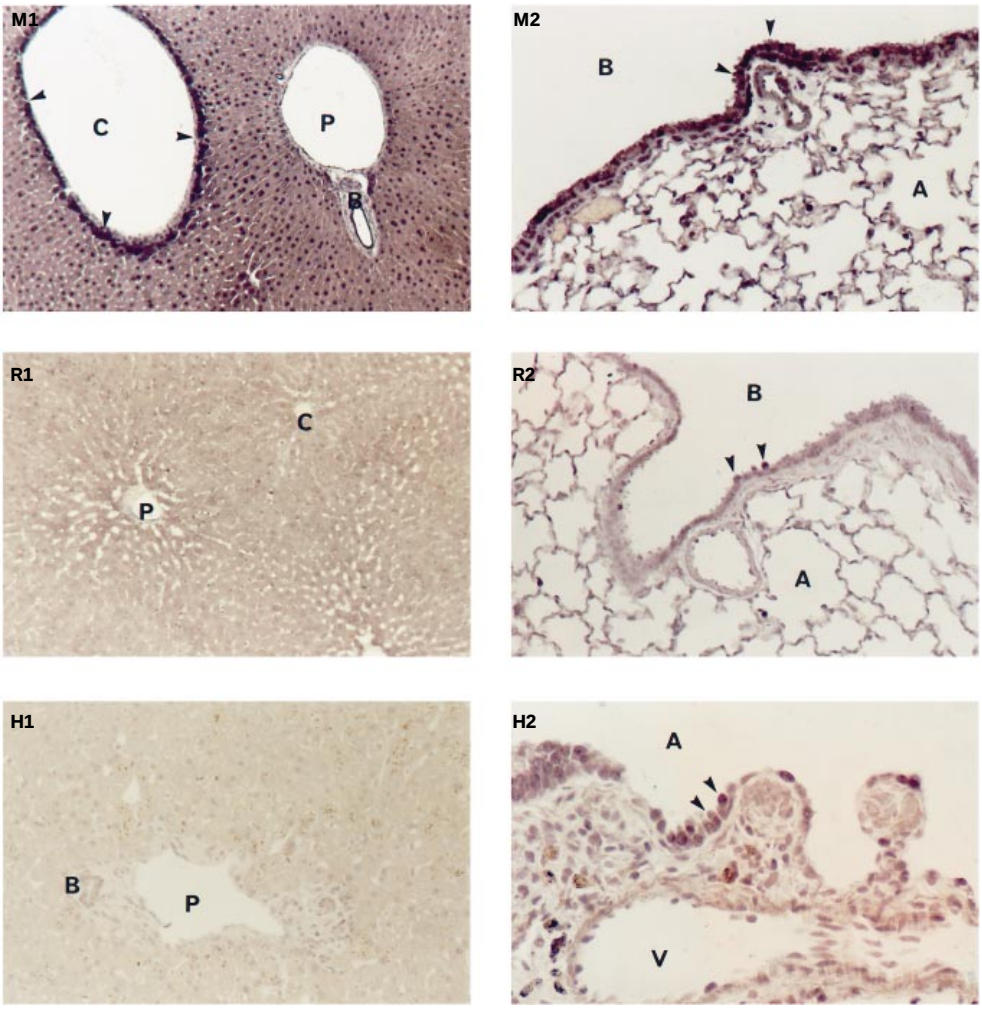

Figure 4 ***In situ*** **hybridization of mRNA to GSTT1-1 in mouse liver (M1) and lung (M2), rat liver (R1) and lung (R2), and human liver (H1) and lung (H2)**

In the lung sections: A corresponds to the alveolus; B, the bronchiolar lumen; and V, a pulmonary blood vessel. Arrows mark the Clara cells. In the liver sections: B corresponds to the bile ducts; C, the central vein; and P the portal vein. Arrows mark the limiting plate hepatocytes in the mouse liver.

alveolar/bronchiolar junction (Figure 4), but were entirely absent from the epithelial cells of the larger bronchioles. The total number of Clara cells in the human lung samples was very low, and their distribution was confined to the immediate alveolar/bronchiolar junction.

When the human lung samples were assayed for their ability to metabolize methylene chloride, a substrate for hGSTT1–1, the rates were extremely low: 0.06, 0.21 and 0.23 $nmol \cdot min^{-1} \cdot mg^{-1}$ of protein, which is approx. an order of magnitude lower than that found in human liver [24].

DISCUSSION

This study has demonstrated a highly specific distribution of Theta-class GSTT1–1 and GSTT2–2 in liver and lung tissue from mice, rats and humans. The techniques of *in situ* hybridization and immunocytochemistry are at best semi-quantitative and were used primarily to study the distribution of these enzymes, yet it was very apparent from these studies that both the distribution and concentration of these enzymes differed markedly between the three species. Whilst neither mRNA levels nor protein concentrations necessarily correspond to active enzyme, the distribution shown by the mRNA for GSTT2–2 was quantitatively reflected by the antibody to the protein of this enzyme, suggesting that these techniques do, in this case, reflect the distribution of active enzyme. Although an antibody to GSTT1–1 is not available, it is reasonable to assume that mRNA levels for this enzyme are similarly representative of the distribution of active enzyme. In support of this assumption, the mRNA for GSTT1–1 in the mouse tissues is far higher than in the other species, as is the rate of metabolism of methylene chloride, a substrate for GSTT1–1 [7,25]. This conclusion is also consistent with the finding that the mouse and rat GSTT1–1 enzymes have similar specific activities [4,7], the high metabolic rates measured in mouse tissues being a result of increased expression of this enzyme rather than a higher affinity for the substrate.

The normal role for glutathione and the glutathione S-transferase enzymes is one of protecting cells from a wide range of chemical and biochemical damage. It is also now recognized that a number of chemicals are metabolized by glutathione conjugation to reactive electrophiles, which can react covalently with cellular macromolecules, including DNA [8,26]. The dis-

tribution of the Theta enzymes in mouse liver cells around the central vein, in the bile-duct epithelium and in nuclei can therefore either preferentially protect these cells or place them at risk. In the case of methylene chloride, a mouse liver and lung carcinogen which is metabolized to a reactive glutathione conjugate by mouse Theta enzyme mGSTT1–1, the latter is clearly the case. It is also logical to assume that the cells most at risk from DNA reactive, but transient, electrophiles are those cells with high concentrations of Theta enzymes in the nucleus. Interestingly, not all hepatocytes had nuclei which contained Theta GSTs. Even more surprisingly, in several binucleate cells only one of the nuclei stained for the enzyme (Figure 3). GSTs of the Theta class and of the main GST families have been reported in nuclei in the past [27–29], although their exact role in the nucleus is not fully understood. They have been associated with peroxidase activity, with the repair of oxidative DNA damage and with DNA-binding proteins with a role in cell division [27,30]. The current observations, particularly that concerning binucleate cells, suggests that this area is worthy of further study.

In mouse liver, the distribution of GSTT1–1 and GSTT2–2 was essentially the same, whether determined as mRNA or as protein. In rat and human liver, GSTT1–1 mRNA was present at considerably lower levels and showed no preferential distribution in any cell type, nor was this enzyme localized in the nuclei. The low levels of this enzyme in rat liver, its general distribution, and in particular its absence from nuclei is consistent with the lack of a tumorigenic response in rats exposed to the mouse carcinogen methylene chloride [9]. On the basis of a similar distribution and even lower levels in human liver, it would appear highly unlikely that methylene chloride would be a human liver carcinogen. In contrast to GSTT1–1, the distribution of GSTT2–2 in rat liver was comparable to that in the mouse, albeit at lower concentrations. The significance of this observation is uncertain, since the substrate specificity of rGSTT2–2 is currently poorly characterized. The nucleotide sequence for the human equivalent of this enzyme (hGSTT2–2) was not available when this work was conducted, and consequently a probe for this enzyme could not be made. This sequence has subsequently been published [31]. Using the rat GSTT2-2 probe and antibody, the levels of this enzyme in human liver were very low and showed a general distribution throughout the lobule. The antibody did however reveal high concentrations of this enzyme in the bile ducts of human liver.

The highest concentrations of Theta enzymes in the lungs of mice were found in the bronchiolar epithelium, principally in Clara cells and ciliated cells where the levels were at least comparable to the highest concentrations found in mouse liver, if not even higher. The type II cells of the lung parenchyma also had low levels of both enzymes. It was not possible to distinguish the nuclei of mouse-lung Clara cells and ciliated cells because of the heavy and uniform staining over the whole cell, suggesting that, as in the mouse liver, the enzymes were present in the nuclei of these cells. The amounts of both enzymes in rat lung were considerably lower than those in mouse lung and were restricted to Clara cells only. As in the liver, the marked difference in the levels of GSTT1–1 between rat and mouse lung is consistent with the species difference in the carcinogenicity of methylene chloride. The cell of origin of the mouse lung tumours following exposure to methylene chloride remains uncertain. Based on the highest concentrations of the activating GSTT1–1 enzyme, either Clara cells or ciliated cells could fulfil this role.

In human lung, the rGSTT2–2 antibody showed low levels of protein in the bronchiolar epithelium, comparable to those seen in the rat. GSTT1–1, on the other hand, appeared to be completely absent from the large bronchioles and was only found in a few Clara cells and ciliated cells at the alveolar/bronchiolar junction. This finding also confirmed that the number of Clara cells in human lungs are very few in comparison to either the rat or mouse where they are found throughout the airways. The apparent lack of hGSTT1–1 in some of the lung samples is probably related not to the polymorphism known to be present in the human population with this enzyme [32,33], but to the very low intrinsic levels which were at, or below, the limit of detection of the technique. Consistent with this were the very low rates of metabolism of methylene chloride in human lung fractions.

In conclusion, the distribution and levels of Theta-class GSTs have been found to differ significantly between the liver and lungs of the three species studied. The molecular and biochemical basis of the cellular distribution or the nuclear localization in mouse tissues is not currently understood. An understanding of the cellular and sub-cellular distribution of GSTT1–1 has provided an explanation for the species specificity of the mouse lung and liver carcinogen methylene chloride, and has provided reassurance that humans are not at risk from exposure to this chemical. This approach clearly leads to a greater understanding of species differences in response to chemicals than the more usual methodology of comparing metabolic rates in tissue homogenates or crude cell preparations.

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