Characterization of Phenotypes in \textit{Gstm1}-null Mice by Cytosolic and in Vivo Metabolic Studies Using 1,2-Dichloro-4-Nitrobenzene

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\textbf{ABSTRACT:}

Glutathione S-transferase Mu 1 (GSTM1) has been regarded as one of the key enzymes involved in phase II reactions in the liver, because of its high expression level. In this study, we generated mice with disrupted glutathione S-transferase Mu 1 gene (\textit{Gstm1}-null mice) by gene targeting, and characterized the phenotypes by cytosolic and in vivo studies. The resulting \textit{Gstm1}-null mice appeared to be normal and were fertile. Expression analyses for the \textit{Gstm1}-null mice revealed a deletion of \textit{Gstm1} mRNA and a small decrease in glutathione S-transferase alpha 3 mRNA. In the enzymatic study, GST activities toward 1,2-dichloro-4-nitrobenzene (DCNB) and 1-chloro-2,4-dinitrobenzene (CDNB) in the liver and kidney cytosols were markedly lower in \textit{Gstm1}-null mice than in the wild-type control. \textit{Gstm1}-null mice had GST activities of only 6.1 to 21.0\% of the wild-type control to DCNB and 26.0 to 78.6\% of the wild-type control to CDNB. After a single oral administration of DCNB to \textit{Gstm1}-null mice, the plasma concentration of DCNB showed larger AUC\textsubscript{0–24} (5.1–5.3 times, versus the wild-type control) and higher \textit{C}_{\text{max}} (2.1–2.2 times, versus the wild-type control), with a correspondingly lower level of glutathione-related metabolite (AUC\textsubscript{0–24} 9.4–17.9\%; and \textit{C}_{\text{max}} 9.7–15.6\% of the wild-type control). In conclusion, \textit{Gstm1}-null mice showed markedly low ability for glutathione conjugation to DCNB in the cytosol and in vivo and would be useful as a deficient model of GSTM1 for absorption, distribution, metabolism, and excretion/toxicology studies.

Glutathione S-transferases (GSTs) form a superfamily that is characterized by catalysis of the conjugation of glutathione (GSH) with various electrophilic compounds (Hayes and Pulford, 1995). At least seven distinct classes (Alpha, Mu, Pi, Sigma, Theta, Kappa, and Zeta) of soluble GSTs have been identified so far, according to substrate specificity, chemical affinity, structure, and the kinetic behavior of the enzyme (Landi, 2000; Strange et al., 2000). However, a large number of GST isoforms that overlap the substrates’ specificities and broad GST expression in various tissues do not encourage the characterization of the properties for each GST isoform in the whole body.

The use of knockout mice with one disrupted gene among a large number of xenobiotic-metabolizing enzyme isoforms shows great advantages in understanding the pharmacokinetics of xenobiotics. Three knockout mice lines for GST isoforms, \textit{Gsta}4 KO mice (Engle et al., 2004), \textit{Gstpl}p2 KO mice (Henderson et al., 1998, 2000; Elsby et al., 2003), and \textit{Gstz}1 KO mice (Fernandez-Canon et al., 2002; Lim et al., 2004), have been established so far. However, the pharmacokinetics of the specific substrate for the GST isoform in these knockout mice lines has not been analyzed yet, and there are only a few reports about the pharmacokinetics of the specific substrate in the knockout mice with disrupted xenobiotic-metabolizing enzyme genes other than GSTs.

In mouse, GSTA3, GSTP1, and GSTM1 show high expression levels in the liver and kidney, and have been regarded as the key enzymes for phase II reactions and the detoxification of carcinogens/ mutagens, environmental pollutants, and anticancer drugs (Mitchell et al., 1997). In this study, we focused on GSTM1, which is one of six Mu class GST isoforms in mice (Hayes and Pulford, 1995; Guo et al., 2002), and generated mice with disrupted glutathione S-transferase Mu 1 gene (\textit{Gstm1}-null mice) by gene targeting. We characterized the phenotypes by in vitro and in vivo metabolic studies.

\textbf{Materials and Methods}

\textbf{Generation of \textit{Gstm1}-null Mice.} To construct the targeting vector, DNA from W9.5 ES cells was used to amplify \textit{Gstm1} genomic fragments for both the 5’ and 3’ arms. For the 5’ arm, a 6.8-kb 5’-flanking sequence of the \textit{Gstm1} gene was cloned into the Hpal and Xhol sites of the pKO Scrambler V901 plasmid. For the 3’ arm, a 3.0-kb 3’-flanking sequence of the \textit{Gstm1} gene was

\textbf{ABBREVIATIONS:} GST, glutathione S-transferase; GSTM1, glutathione S-transferase Mu 1; GSTa or GSTa, glutathione S-transferase Alpha; Gstp, glutathione S-transferase Pi; Gstz, glutathione S-transferase Zeta; GSH, glutathione; KO, knockout; ES cell, embryonic stem cell; kb, kilobase(s); DT, \textit{diphertheria toxin A chain} gene; RT-PCR, reverse transcription-polymerase chain reaction; DCNB, 1,2-dichloro-4-nitrobenzene; CDNB, 1-chloro-2,4-dinitrobenzene; GST-D activity, GST activity toward DCNB; GST-C activity, GST activity toward CDNB; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-tandem mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ESI, electrospray ionization; ASK1, apoptosis signal-regulating kinase 1; bp, base pair(s).
cloned into the SacII and SalI sites. The primers for the 5' arm were 5'-AGTTGATAACATCTTTATCTTCCGACATC-3' and 5'-CCCTCTGAGAGTTACAGGAACAGTCGGTGACATG-3'. The primers for the 3' arm were 5'-AGACCCGGGGGTCTTCCACCTGGACATGCCAC-3' and 5'-CGGCTGACACAGACTCTTTGGAAGAGATGTGGTGG-3'. For the positive-negative selection, a neomycin-resistant (Neo') cassette from the pKO SelectNeo V800 plasmid and diptheria toxin A chain gene (DT) from pKO SelectDT V840 plasmid was used. All plasmids were supplied from TAKARA Bio Inc. (Oshtsu, Japan). W9.5 ES cells (10^7) were electroporated (250 V, 500 μF) with 20 μg of the linearized targeting vector and selected with 250 μg (active form)/ml G418 for 8 to 10 days (Fujimoto et al., 2006). G418-resistant clones were primarily screened on the 35 ml G418 for 8 to 10 days (Fujimoto et al., 2006). G418-resistant clones were twice checked on the 50 μg/ml G418. The PCR-positive clones were twice selected on the 50 μg/ml G418. PCR analyses of the homologous recombination on the 5' and 3' arm, respectively, B, PCR analyses of the homologous recombination on the 5' and 3' arm. GM2H/Neo3 primer set was amplified by a 3.1-kb fragment contained in the 3' arm on the mutant allele (left). Neo3/M1R2 primer set was amplified by a 3.1-kb fragment contained in the 3' arm on the mutant allele (right). Lambda/HindIII digest was used as a molecular size marker. wt, wild type.

Measurement of the GST Activities Toward CDNB and DCNB in the Cytosols of the Liver and Kidney. The liver and kidney samples of the Gstm1-null mice, the heterozygotes, and the wild-type controls were mixed with 1.15% potassium chloride (1:3, w/v) and homogenized in an ice bath. The homogenates were centrifuged at 9000 g for 20 min at 4°C, and the supernatant fraction was further centrifuged at 105,000 g for 1 h at 4°C to isolate the cytosolic fraction. Protein concentrations in the cytosolic fractions were determined by the method of Lowry et al. (1951). The GST activity toward CDNB (GST-C activity) was measured according to the established method (Habig et al., 1974). Briefly, the cytosol was diluted 30-fold with 1.15% potassium chloride, and 0.3 ml of 20 mM GSH and 0.1 ml of the diluted cytosol were mixed in 5.3 ml of 100 mM potassium phosphate buffer (pH 6.5). After the addition of CDNB at a final concentration of 1 mM, the change in absorbance at 345 nm was measured for 1 min with a spectrophotometer. The GST activity toward DCNB (GST-D activity) was measured according to the method of Lowry et al. (1951). The GST activity toward CDNB (GST-C activity) was measured according to the established method (Habig et al., 1974). Briefly, the cytosol was diluted 30-fold with 1.15% potassium chloride, and 0.3 ml of 20 mM GSH and 0.1 ml of the diluted cytosol were mixed in 5.3 ml of 100 mM potassium phosphate buffer (pH 7.5). After addition of DCNB at a final concentration of 1 mM, the change in absorbance at 345 nm was measured for 1 min with a spectrophotometer.
Measurement of Plasma DCNB and the Metabolites Concentrations After Administration of DCNB. Gstm1-null mice and the wild-type controls were treated via oral administration with 1% DCNB dissolved in polyethylene glycol at dose levels of 100 mg/kg. Approximately 0.3 ml of blood was collected at 30 min, and 1, 2, 4, 8, and 24 h postdose. The blood samples were centrifuged at 10,000 rpm for 5 min at 4°C to prepare the plasma samples. Then, 50 μl of each plasma sample was mixed with 150 μl of ethanol and centrifuged at 3000 rpm for 5 min at 4°C. To determine the plasma concentration of DCNB, the supernatant was subjected to a high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) consisting of a system controller (SCL-10A), a pump (LC-10AD), an autosampler (SIL-10AC), a column oven (CTO-10AC), and a UV detector (SPD-10A). The HPLC conditions for these analyses were as follows: the column was an L-column ODS (150 mm × 4.6 mm i.d.; Chemicals Evaluation and Research Institute, Tokyo, Japan); the column temperature was 40°C; mobile phase was water/acetonitrile/ammonium acetate (1 M) at 65:35:1 (v/v/v); flow rate was 1.0 ml/min; injection volume was 20 μl; and UV detector wavelength was 270 nm.

LC-MS and LC-MS/MS Analyses. LC-MS and LC-MS/MS analyses were performed using a Q-Tof mass spectrometer (Waters, Manchester, UK) with an HPLC system (Hitachi High-Technologies Corp., Tokyo, Japan) consisting of an intelligent pump (model L-7100), a column oven (model L-7300), a chromatographic integrator (model D-7500), and a UV detector (model L-7400S). The LC-MS analysis was conducted using electrospray ionization (ESI) in the positive ion mode. The capillary voltage and cone voltage were set at 3300 V and 45 V, respectively. The source temperature and desolvation gas temperature were 120°C and 300°C, respectively. The mass range from m/z 50 to 900 was acquired with an integration time of 1 s. The LC-MS/MS experiments were performed using a collision energy of 20 eV and xenon as the collision gas. The chromatographic separation was carried out on a YMC-Pack ODS-A 312 column (6.0 × 150 mm; YMC Corporation, Kyōto, Japan), the column temperature was maintained at 40°C, and UV detection was at 270 nm. The mobile phase was used was water/acetonitrile/ammonium acetate (1 M) at a ratio of 82:18:1 (v/v/v). The flow rate was set at 0.1 ml/min and the elution flow from HPLC was introduced into the Q-Tof ionization source through an ESI interface.

Statistical Analysis. The values of the mean and the standard deviation in each group were calculated with statistical software (SAS System Version 5.0; SAS Institute Inc., Cary, NC). A parametric Dunnett’s test for the mean value used was performed to analyze the differences between the wild-type control and the hetero/homozygote. A value of P < 0.05 was chosen as an indication of statistical significance.

Results

Generation of Gstm1-null Mice. Mice with disrupted Gstm1 gene were generated by a homologous recombination method with a mouse ES cell. The targeting vector used in this study is shown in Fig. 1A. Mouse Gstm1 gene consists of eight exons in a region of approximately 5.7 kb. This targeting vector was designed to achieve the deletion of the entire coding region of the Gstm1 gene by replacing it with a Neo’ cassette. W9.5 ES cells were electroporated with the targeting vector, and 288 of the colonies with G418 resistance were selected and screened by PCR for homologous recombination. Two independent PCR-positive clones, 4B3 and 10C4, were obtained by primary PCR screening with a NeoF3/M1R2 primer pair (Fig. 1B, right). However, secondary PCR screening with a GM2H/NP3 primer pair revealed that only the 4B3 clone was integrated into the targeted sequence by homologous recombination (Fig. 1B left). The 4B3 ES cells were aggregated with two zona pellucida-free eight-cell embryos from C57BL/6J mice and cultured for 24 h. Aggregated blastocysts were transferred into the uterus of the surrogate mothers. Chimera mice were identified by coat color and crossed to C57BL/6J mice, and F2 heterozygous offspring to which the targeted ES cells had contributed were identified by coat color and PCR genotyping. Furthermore, the F2 heterozygous mice were intercrossed to produce F2 mice, which were determined to be wild-type (+/+), heterozygotes (+/−), or homozygotes (−/−; Gstm1-null mice) by PCR genotyping (Fig. 2A).

Gstm1-null mice appeared to be normal and were fertile as the wild-type and heterozygote. No obvious histological, hematological, and blood chemical differences in the basal condition were detected between Gstm1-null mice and the wild-type control (data not shown).

Gstm1, Gsta3, and Gstp1 mRNA Expression Analysis. Gstm1 mRNA expression was not detected in the livers of Gstm1-null mice by RT-PCR (Fig. 2B). This result indicates a disruption of the Gstm1 gene in Gstm1-null mice. Furthermore, to confirm whether Gstm1 gene disruption has an effect on expression levels in the livers of Gstm1-null mice, real-time quantitative RT-PCR was performed (Fig. 2B). These results revealed slightly low Gsta3 and Gstpl mRNA expression, the expression levels of which were determined to be 64.7% and 86.5% of the wild-type control, despite the same levels of Gsta1 mRNA expression in the livers of Gstm1-null mice (Fig. 3).

GSTM-C and GST-D Activity in the Cytosol of the Liver and Kidney. We measured the GST-C and GST-D activity in cytosolic samples from the livers and kidneys of Gstm1-null mice. The results revealed low GST-C and GST-D activity in the Gstm1-null mice (Fig. 4). GST-C activity was slightly low in the liver of the males (78.6% of the wild-type control), and markedly low in the liver of females (32.3% of the wild-type control) and in the kidneys of both sexes (male, 26.0%; female, 33.8% of the wild-type control). GST-D activity was nearly absent in the livers and the kidneys of both sexes (male liver, 9.9%; female liver, 8.7%; male liver, 9.0%).
kidney, 6.1%; female kidney, 21.0% of the wild-type control). The heterozygotes showed intermediate GST-C and GST-D activity between that of the homozygotes and wild-types in the all-cytosolic samples.

Plasma Concentrations of DCNB and Its Metabolite, M0, After Administration of DCNB. We measured the plasma concentrations of DCNB after a single oral administration of DCNB (100 mg/kg) to wild-type and Gstm1-null mice by HPLC, to evaluate the pharmaco-kinetis of DCNB in the whole body. This result is shown in Fig. 5. The AUC0–24 was 6.04 ± 0.62 µg·h/ml in the male wild-type control, 31.72 ± 9.44 µg·h/ml in the male Gstm1-null mice, 9.02 ± 1.27 µg·h/ml in the female wild-type control, and 45.99 ± 5.34 µg·h/ml in the female Gstm1-null mice. The Cmax was 1.76 ± 0.53 µg/ml in the male wild-type control, 3.73 ± 0.62 µg/ml in the male Gstm1-null mice, 1.95 ± 0.23 µg/ml in the female wild-type control, and 4.30 ± 0.22 µg/ml in the female Gstm1-null mice. After a single oral administration of DCNB to Gstm1-null mice, the plasma concentration of DCNB showed a larger AUC0–24 (male, 5.3 times; female, 5.1 times, versus the wild-type control) and higher Cmax (male, 2.1 times; female, 2.2 times, versus the wild-type control). Furthermore, three peaks were determined in the plasma after administration of DCNB to the wild-type control, as major metabolite peaks in the HPLC chromatogram. As shown in Fig. 6, the concentration of M0, which had the highest level among the three metabolites, was lower in the plasma of Gstm1-null mice. After a single oral administration of DCNB, Gstm1-null mice showed smaller AUC0–24 (male, 9.4%; female, 17.9% of the wild-type control) and lower Cmax (male, 9.7%; female, 15.6% of the wild-type control).

Structure Analysis of Metabolite M0. The DCNB metabolite, M0, was detectable in mouse plasma. The molecular-related ion [M + H]+ of M0 was observed at m/z 248 in the positive ion mode. Thus, the molecular weight of metabolite M0 was proposed to be 247. The LC-MS/MS spectrum of the precursor ion [M + H]+ at m/z 248 and major fragment ions are shown in Fig. 7. The fragment ion at m/z 206 was formed via elimination of the acetyl moiety from the ion at m/z 248. The fragment ion at m/z 170 was formed via elimination of the
Discussion

We generated mice with disrupted Gstm1 gene, which is expressed predominantly in the liver, by homologous recombination, to investigate the properties of mouse GSTM1 in xenobiotic metabolism. An ES cell clone with the predicted mutant allele, confirmed by genomic PCR and sequencing analyses, was used to generate mice with disrupted Gstm1 gene. RT-PCR analysis revealed a lack of Gstm1 mRNA expression in the homozygote. Furthermore, two-dimensional gel electrophoresis/mass spectrometry analyses also revealed that GSTM1 protein expression was completely absent in the homozygotes (data not shown). These results indicated success in the generation of the Gstm1-null mice.

In the enzymatic study, GST-D activity was nearly absent in the livers and kidneys of both sexes in Gstm1-null mice. Mouse GSTM1 purified from liver cytosol has been reported to specifically catalyze DCNB (Warholm et al., 1986). The present study also clarified that DCNB is a specific substrate for mouse GSTM1. Conversely, Gstm1-null mice showed low GST-C activity (approximately 30% of the wild-type control) in the kidneys of both sexes and in the female livers, and a slightly low GST-C activity (approximately 80% of the wild-type control) in the male livers.

Interestingly, GST-C activities with broad specificity to various GSTs were lower than our expectation in the male and female kidneys and the female livers of the Gstm1-null mice, indicating that GSTM1 is the main enzyme to catalyze CDNB metabolism in mouse male and female kidneys and female livers. The higher hepatic GST-C activity in the male Gstm1-null mice may be due to the higher expression of pi class GSTs in males than females (Mitchell et al., 1997).

Plasma DCNB concentrations after an administration of DCNB to Gstm1-null mice showed larger AUC0–24 (male, 5.3 times; female, 5.1 times, versus the wild-type control) and higher Cmax (male, 2.1 times; female, 2.2 times, versus the wild-type control), consistent with lower GST-D activity in vitro. In the same way, the concentration of M0, the major metabolite among the three DCNB metabolites separated on the HPLC chromatogram, showed a smaller AUC0–24 in the plasma (male, 9.7%; female, 15.6% of the wild-type control) and higher Cmax (male, 9.7%; female, 15.6% of the wild-type control) in Gstm1-null mice. This suggested that M0 might be a GSH-related metabolite of DCNB. Actually, the structure analysis by LC-MS/MS revealed that M0 was the GSH-related metabolite, the methylsulfone moiety from the ion at m/z 248. As well, a high resolution LC-ESI/MS analysis demonstrated the elemental composition of the molecular-related ion [M + H]+ at m/z 248 to be C9H11NO3SCl (data not shown), which supports the fact that metabolite M0 is a methylsulfone-N-acetyl form of DCNB. Therefore, the chemical structure of M0 was found to be a methylsulfone-N-acetyl form produced by the glutathione conjugation of the DCNB followed by several metabolic reactions.

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et al., 2004; Lim et al., 2004). In Gstm1-null mice, a slightly low expression level of Gsta3 mRNA was observed by the GeneChip microarray and real-time quantitative RT-PCR analyses, despite there being no change in the Gstp1 mRNA expression level. It has actually been reported that mouse GSTA3 has low GST-C and GST-D activities (Hayes and Pulford, 1995). From these results and available information, the slight down-regulation of the Gsta3 mRNA expression might be involved in the low GST-C and GST-D activities in Gstm1-null mice established in this study.

GSTs are important enzymes for xenobiotic metabolism and toxic phenotype. However, a large number of GST isoforms overlap the substrates’ specificities, and a broad GST expression in various tissues could not facilitate the characterization of the properties of each GST isoform in the whole body. The GST knockout mice would facilitate the investigation of these properties and help to understand the metabolic pathways through the GST isoforms (Hayes et al., 2005). However, only a few reports on GST knockout mice have been published so far. In the present study, we generated knockout mice for the Gstm1 gene and clarified that the GSTM1 deficiency results in markedly low GST-D activity both in vitro and in vivo. Furthermore, it has been reported that mouse GSTM1 inhibits apoptosis signal-regulating kinase 1 (ASK1) by forming GSTM1/ASK1 complex and mediates apoptosis in response to heat shock stress (Cho et al., 2001; Dorion et al., 2002). These observations suggest that GSTM1 is regulating kinase 1 (ASK1) by forming GSTM1/ASK1 complex and inhibiting apoptosis signal-regulating kinase 1.

In conclusion, we generated knockout mice for the Gstm1 gene and found that GST-D activity in the liver and kidney cytosols markedly decreased in Gstm1-null mice, and that a single oral administration of DCNB to Gstm1-null mice resulted in larger AUC and higher Cmax for the plasma concentration of DCNB, and smaller AUC and lower Cmax for the plasma concentration of M0, a GSH-related metabolite of DCNB. This mouse model would be useful as an animal model exhibiting poor metabolism of the specific substrates to mouse GSTM1 for absorption, distribution, metabolism, and excretion/toxicology study.

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